REMARKS

I. Preliminary Comments and Amendments to the Specification

Applicants note, with gratitude, the withdrawal of the previous rejections and the entry of the amendment correcting the obvious typographical error.

Applicants hereby amend independent claim 6 in accordance with the suggestion of the Examiner to delete the recitation of "correlating said change in expression level, mutation or rearrangement with a standard indicative of a hyperproliferative disease to determine the occurrence of a hyperproliferative disease" and recite in its place "wherein said change in expression level, mutation or rearrangement is indicative of hyperproliferative disease." No new matter is introduced by this amendment, and it is submitted that the amendment places the claims in condition for allowance.

II. Subject Matter of the Invention

The present application relates to the discovery that the ELP protein is a tumor suppressor (see page 6, lines 3-30, and Example X, page 35, lines 1-30), and that alterations in expression levels of ELP, mutations in the nucleic acid sequence encoding an ELP protein, or rearrangements in the genomic *elp* locus correlate with altered tumor suppressor activity and, consequently, with hyperproliferative diseases or a predisposition thereof. (*See* page 9, line 31 – page 11, line 9). In addition, Applicants have provided methods of analyzing samples of subjects to identify abnormalities in ELP, either in protein or mRNA expression or in mutations of the DNA sequence, as a means of identifying a hyperproliferative disease. (*See* Example XI, page 35, line 33 – page 36, line 22).

III. Outstanding Rejections

Claims 6-12 stand rejected under 35 U.S.C. §112, first paragraph, for lack of written description.

Claims 6-12 stand rejected under 35 U.S.C. §112, first paragraph, for lack of enablement with respect to detection in body fluid samples.

IV. Patentability Arguments

A. Rejection of Claims 6-12 under 35 U.S.C. §112, First Paragraph (written description).

The rejection of claims 6-12 for lack of written description/new matter should be withdrawn in light of the amendment of independent claim 6 to delete the recitation of "correlating said change in expression level, mutation or rearrangement with a standard indicative of a hyperproliferative disease to determine the occurrence of a hyperproliferative disease" and substitution in its place of the language "wherein said change in expression level, mutation or rearrangement is indicative of hyperproliferative disease" as suggested by the Examiner. No new matter is introduced thereby, and it is submitted that the rejection for lack or written description/new matter for the language previously submitted may be withdrawn.

B. Rejection of Claims 6-12 under 35 U.S.C. §112, First Paragraph (enablement).

Applicants note, with appreciation, the withdrawal of the previous rejections (including rejections for lack of enablement) and the acknowledgement by the Examiner at page 4 of the Action that the disclosure is "enabling for a method of the identification of a hyperproliferative disease, which comprises detecting a change in ELP mRNA or protein expression levels from a tissue sample..."

Applicants submit that their disclosure also enables those of ordinary skill in the art to practice the method of identification of a hyperproliferative disease in a body fluid. While the specification provides no examples for measuring *Elp* gene sequence or expression at the RNA or protein levels in bodily fluids, such methods were well known in the art and are expected to function according to Applicants' invention. Not only does the scientific literature show that the analysis of bodily fluids for the diagnosis of non-hematopoetic tumors was well accepted but the specific concerns voiced in the Action that the tumor cell antigen expression might be down-regulated or lost are misplaced.

The art teaches that cells disseminate from non-haematopoetic tumors and can be found in serum and bone marrow as well as other bodily fluids. There were (and are) numerous publications confirming the presence and the validity of the detection of tumor

cells in bodily fluids. Such cells can obviously resist for a long time and can be the cause of a spreading of the cancer through formation of metastasis. Two examples of such publications impressively demonstrate the presence of disseminated cells from non-haematopoetic tumors in the bodily fluids:

Kasimir-Bauer et al., 2001: This group investigated the presence of disseminated breast cancer cells even after high-dose chemotherapy and indeed such cells could be found in the bone marrow.

Lauschke et al., 2001: This group investigated the presence of mutations in the oncogene K-ras and the tumor suppressor gene APC in *serum* DNA of colon cancer patients. Whereas, K-ras (oncogene) mutations were difficult to find in serum, APC (tumor suppressor gene) mutations were detected in 80% (20/25) in the serum of patients with APC mutations in the primary tumor. Beside this significant result, the group could detect the presence of mutant DNA in the serum 10 days after surgical removal of the tumor which led them to conclude that cells or at least mutant DNA of disseminated cells are not rapidly cleared from the blood.

Submitted herewith is the Declaration of Barbara Froesch, Ph.D. (Exhibit 1) stating that it was well established at the time of applicants' invention that analysis of bodily fluids was a reliable measure to diagnose tumors. A review of the scientific literature demonstrates that the analysis of bodily fluids such as saliva, serum, urine, peritoneal fluid and bone marrow for DNA and other markers could be used for the diagnosis of non-haematopoetic tumors like lung, renal and gastric cancers as set out in the publications below:

Publication	**Set-up**	**Result**
Hibi et al., "Molecular Detection of Genetic Alternations in the Serum of Colofectal Cancer Patients," *Cancer Research* 58: 1405-1407 (1998)	Microsatellite analysis of genetic alterations in **serum DNA** obtained from 44 **colorectal cancer** patients.	"Taken together, either a K-*ras* or p*53* mutation was detected in the serum in 40% of the 25 patients (95% confidence interval, 21-61%), whose primary tumors contained a mutation and in 23% of the 44 patients (95% confidence interval 12-38%) with colorectal cancer. The frequent detection of *p53*

		mutation in the serum of patients with early stage tumor suggests a possible use of this approach for clinical prognosis and cancer monitoring of colorectal cancer patients."
El-Naggar et al., "Genetic heterogeneity in saliva from patients with oral squamous carcinomas:implications in molecular diagnosis and screening," *J Mol Diagn.* 3(4):164-70 (2001).	Microsatellite analysis of **head and neck squamous carcinoma** (HNSC) on **saliva** and matched tumors in 37 patients.	-"epithelial cells in saliva[...]provide suitable material for genetic analysis" -in 49% of patients genetic alterations (LOH) could be detected in saliva
Spafford et al., "Detection of head and neck squamous cell carcinoma among exfoliated oral mucosal cells by microsatellite analysis," *Clin Cancer Res.* 7(3):607-12 (2001).	Microsatellite analysis of **saliva** in **head and neck squamous cell carcinoma** (HNSCC) patients.	Microsatellite instability was detected in 96% and LOH in 61% of cases with corresponding genetic alterations in the primary tumors.
Lauschke et al., "Detection of APC and k-ras mutations in the serum of patients with colorectal cancer," *Cancer Detect Prev.* 25(1):55-61 (2001).	Detection of K-ras and APC mutations in **serum DNA** of **colon** cancer patients.	K-ras mutations only found in the serum of 6/22 patients but APC mutations found in serum of 20/25 patients
Schott et al., "Isolated tumor cells are frequently detectable in the peritoneal cavity of gastric and colorectal cancer patients and serve as a new prognostic marker," *Ann Surg.* 227(3):372-9 (1998).	**Bone marrow** or **peritoneal cavity fluid** of **gastric** or **colorectal** patients were investigated immunocytochemically.	Little prognostic significance of positive bone marrow results but high correlation of results from peritoneal cavity fluid and survival rate.

Kersting et al., "Differential frequencies of p16(INK4a) promoter hypermethylation, p53 mutation, and K-ras mutation in exfoliative material mark the development of lung cancer in symptomatic chronic smokers," *J Clin Oncol.* 18(18):3221-9 (2000).	**Sputum** analysis in **lung cancer** patients for p53, K-ras and p16.	Differences on DNA level could be detected in 14-51% of patients if the markers were analyzed individually but in 69% if the markers were combined.
Eisenberger et al., Diagnosis of renal cancer by molecular urinalysis," *J Natl Cancer Inst.* 91(23): 2028-32 (1999).	Microsatellite alterations in **urine** and **serum** of **renal cancer** patients.	Alterations could be found in 76% of urine samples and 60% of serum samples.
Vogel et al., "Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor," *Clin Cancer Res.* 5(3):593-9 (1999).	Immunocytochemical analysis of **peritoneal cavity fluid** and **bone marrow** in **pancreatic cancer** patients.	In 39 and 38% of patients alterations could be found in peritoneal lavage or bone marrow, respectively. Combination of the two bodily fluids resulted in 52% of correctly diagnosed patients.
Weitz et al.,"Detection of disseminated colorectal cancer cells in lymph nodes, blood and bone marrow," *Clin Cancer Res.* 5(7):1830-6 (1999).	Expression analysis of CK by RT-PCR on **lymph nodes** (lymphatic system), **blood** and **bone marrow** samples of **colon cancer** patients.	CK detection in lymph nodes is of prognostic relevance.
Chang et al., "Molecular diagnosis of primary liver cancer by microsatellite DNA analysis in the serum," *Br J Cancer.* 87(12): 1449-53 (2002).	Analysis for LOH in **serum of liver cancer** patients.	LOH found in 76.2% of patients.

Hickey et al., "Molecular detection of tumour DNA in serum and peritoneal fluid from ovarian cancer patients," *Br J Cancer* 80(11):1803-8 (1999).	Analysis of genetic alterations in **serum** and **peritoneal fluid** of **ovarian cancer** patients.	In 17/20 serum samples and 12/19 peritoneal fluid samples, genetic alterations could be detected.
A. Vogel et al., "Disseminated tumor cells. Their detection and significance for prognosis of gastrointestinal and pancreatic carcinomas" *Virchows Arch.* 439(2): 109-17 (2001).	Review of the various **methods to detect disseminated tumor cells** used at the time and the prognostic relevance of the results gained from these studies.	"Our evaluation of the studies on colorectal, gastric and pancreatic ductal carcinomas indicates that detection of disseminated tumor cells in different compartments may lead to more accurate tumor staging."

These publications show that analysis of bodily fluids for the diagnosis of non-haematopoetic tumors was well accepted in the art at the time of applicants' invention because disseminated tumor cells or DNA of non-haematopoetic tumor cells could be found in nearly all bodily fluids (e.g., serum, saliva, bone marrow, peritoneal cavity fluid, sputum, urine, lymph nodes, and blood, as noted in the above publications). While negative results exist they depend upon the marker/antigen/genetic alteration that is investigated. A striking example for this is the low significance in diagnosing colorectal cancer by the detection of mutations of the oncogene K-ras in comparison to mutation in the <u>tumor suppressor genes</u> APC or *p53* (see Lauschke et al. and Hibi et al.).

Tumor diagnosis by the analysis of bodily fluids is well accepted and state of the art at the time of applicants' invention. Low performance of certain methods is rather due to poor marker quality and/or tool selectivity (e.g. antibodies) rather than to the method as such. The present invention helps to solve this problem because it introduces a new tumor marker which is significantly downregulated in certain tumors. In addition, in contrast to many other tumor markers (like PSA), the biological function of Elp as tumor suppressor has been shown experimentally (in the disclosure of the specification, at, for example, Example XI at page 35, line 33 – page 36, line 22). Consequently, Elp has already undergone validation as a diagnostic tumor marker.

The enablement rejection on the basis that a protein might not be expressed in the tumor and therefore might not represent a reliable tumor marker does not apply to the present invention which relies upon the widely used method of detecting tumorogenic mutations/rearrangements within the nucleic acid sequence of a tumor suppressor gene like *elp*. The argument that antigen-levels in body fluids are difficult to measure because they are lost or downregulated does not apply for Elp and the present invention because Elp is a tumor suppressor and therefore (already) lost or downregulated in the primary tumor. In the case of Elp, it is therefore very useful as one aspect of the invention to detect mutations of Elp or genomic rearrangements of the Elp locus in disseminated cells which is independent of how reliably one can detect Elp antigen levels.

The reference in the Official Action to Vogel et al. *Virchows Arch* 439:109-117 (2001) (hereafter "Vogel et al.") for the proposition that antigen levels in bodily fluids are hard to measure is said to be supported by Klein et al., who isolated single disseminated cells from bone marrow of a breast cancer patient. An examination of this reference shows, however, that various genetic analyses on those cells comes to a conclusive result despite variances between the single isolated cells (see entire document or last sentence of abstract). They also clearly state that the reliability and variability of such analyses strongly depend on the marker/antigen and the available tools, e.g. antibodies, but less on the methods *per se*. The reviewer further suggests that the problem could be overcome by the parallel use of multiple markers. Thus, the present invention improves the significance of such analyses and therefore aims to solve the problem by the use of an additional tumor marker.

Cells of Non-Haematopoetic Tumors are Present in a Variety of Bodily Fluids

The argument in the Official Action that Vogel et al. (referring to p.110 par 7) teach that "**disseminated tumor cells** of non-haematopoetic origin normally do not circulate in the peripheral blood" is incorrect. Specifically, the Official Action misinterprets the sentence in the review, which cites:

> "When disseminated tumour cells are analysed using RNA-based assays for epithelial cells, it has to be assumed that **cells of non-haematopoetic origin** normally do not circulate in the peripheral blood or one marrow."

The fact that only tumor and not normal non-haematopoetic cells circulate into the peripheral blood or bone marrow represents the prerequisite for the predictive value of such measurements. Otherwise, detection of non-haematopietic cells in bodily fluids could not necessarily be linked to the presence of a tumor. This, however, is not a condition *sine qua non,* as it is only important if the marker *per se* is not tumor specific but primarily a marker for tissue origin and/or differentially expressed in tumor cells without mutations in its nucleic or amino acid sequences. In the case of elp, tumor cell isolation would only be required if one needed to measure elp expression levels, whereas detection of mutations or rearrangements in the elp nucleic acid or protein sequence can directly be investigated without prior isolation of circulating tumor cells, particularly, for instance, of the lung, kidney and stomach. Thus, in case of a tumor suppressor, detection of a mutated nucleic acid or protein sequence in the blood would strongly indicate to a person skilled in the art the presence of a tumor, independently of the concomitant occurrence of tumor cells in the blood.

The state of the art, as outlined in the appended declaration by Dr. Froesch and as summarized in the above table, shows that various cancers have been correlated to a range of markers in bodily fluids. In some cases, the marker is intracellular (e.g., tumor cells from a tissue sample or extracted from a saliva sample), while in other cases, the marker is extracellular (e.g., serum or urine analysis of circulating DNA or protein). The identification of a mutation of the isolated DNA or alteration of expression level of an ELP protein will occur in a similar manner and via means well known in the art, regardless of whether the source of that isolated DNA or ELP protein was intracellular, extracellular, or via a tissue sample.

In conclusion, analysis of body fluids for changes in the expression level of ELP proteins, or mutations within the nucleic acid sequence encoding an ELP protein or detecting a rearrangement in the genomic elp locus was well within the skill of the art given the teachings of the specification and the enablement rejection under 35 U.S.C. § 112, first paragraph, should be withdrawn.

V. Conclusion

In view of the above amendment and remarks, applicants believe the pending application is in condition for allowance. Should the Examiner wish to discuss any issues of

form or substance in order to expedite allowance of the pending application, he is invited to contact the undersigned attorney at the number indicated below.

Dated: September 6, 2006

Respectfully submitted,

By______________________

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EXHIBIT LIST

Exhibit 1 – Declaration of Barbara Froesch Ph.D.

Appendix A - Hibi et al., "Molecular Detection of Genetic Alternations in the Serum of Colofectal Cancer Patients," *Cancer Research* 58: 1405-1407 (1998)

Appendix B - El-Naggar et al., "Genetic heterogeneity in saliva from patients with oral squamous carcinomas:implications in molecular diagnosis and screening," *J Mol Diagn*. 3(4):164-70 (2001).

Appendix C - Spafford et al.," Detection of head and neck squamous cell carcinoma among exfoliated oral mucosal cells by microsatellite analysis," *Clin Cancer Res*. 7(3):607-12 (2001).

Appendix D - Lauschke et al., "Detection of APC and k-ras mutations in the serum of patients with colorectal cancer," *Cancer Detect Prev*. 25(1):55-61 (2001).

Appendix E - Schott et al., "Isolated tumor cells are frequently detectable in the peritoneal cavity of gastric and colorectal cancer patients and serve as a new prognostic marker," *Ann Surg*. 227(3):372-9 (1998).

Appendix F - Kersting et al., "Differential frequencies of p16(INK4a) promoter hypermethylation, p53 mutation, and K-ras mutation in exfoliative material mark the development of lung cancer in symptomatic chronic smokers," *J Clin Oncol*. 18(18):3221-9 (2000).

Appendix G - Eisenberger et al., Diagnosis of renal cancer by molecular urinalysis," *J Natl Cancer Inst*. 91(23):2028-32 (1999).

Appendix H - Vogel et al., "Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor," *Clin Cancer Res*. 5(3):593-9 (1999).

Appendix I - Weitz et al.,"Detection of disseminated colorectal cancer cells in lymph nodes, blood and bone marrow," *Clin Cancer Res*. 5(7):1830-6 (1999).

Appendix J - Chang et al., "Molecular diagnosis of primary liver cancer by microsatellite DNA analysis in the serum," *Br J Cancer*. 87(12):1449-53 (2002).

Appendix K - Hickey et al., "Molecular detection of tumour DNA in serum and peritoneal fluid from ovarian cancer patients," *Br J Cancer* 80(11):1803-8 (1999).

Appendix L – Kasimir-Bauer et al., "Survival of Tumor Cells in Stem Cell Preparations and Bone Marrow of Patients with High-Risk or Metastatic Breast Cancer after Receiving Dose-intensive or High-Dose Chemotherapy," *Clin Cancer Res*. 7:1582-9 (2001).

EXHIBIT 1

Docket No.: 27656/38365A
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Ernst Hafen et al.

Application No.: 10/509,558

Confirmation No.: 1027

Filed: March 25, 2005

Art Unit: 1649

For: GROWTH REGULATING PROTEINS

Examiner: G. S. Emch

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Barbara Froesch, declare the following:

1. I graduated in 1992 from the Swiss Federal Technology Institute (ETH) in Zurich, where I studied pharmaceutical sciences. After the Ph.D. work in the Department of Oncology at the University Hospital of Zurich, I dedicated three years to the study of tumor biology in Dr. John Reed' s laboratory at The Burnham Institute in La Jolla, CA, USA. I joined The Genetics Company, a spin-off of the University of Zurich and the ISREC of Epalinges, as a research scientist in January 1999 and I am currently head of the Biology Department and program manager cancer therapeutics.

2. I have reviewed the Official Action by the U.S. Patent Office dated March 17, 2006 and submit this declaration to address the issue of whether it was recognized at the time of applicants' invention that analysis of bodily fluids was a reliable methodology for diagnosing tumors. A review of the scientific literature demonstrates that the analysis of bodily fluids such as saliva, serum, urine, peritoneal fluid and bone marrow could be used for the diagnosis of non-haematopoetic tumors as set out in the publications below:

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Publication	**Set-up**	**Result**
Hibi et al., "Molecular Detection of Genetic Alternations in the Serum of Colofectal Cancer Patients," *Cancer Research* 58: 1405-1407 (1998)	Microsatellite analysis of genetic alterations in **serum DNA** obtained from 44 **colorectal cancer** patients.	"Taken together, either a K-*ras* or p*53* mutation was detected in the serum in 40% of the 25 patients (95% confidence interval, 21-61%), whose primary tumors contained a mutation and in 23% of the 44 patients (95% confidence interval 12-38%) with colorectal cancer. The frequent detection of mutations in the *p53* tumor suppressor in the serum of patients with early stage tumor suggests a possible use of this approach for clinical prognosis and cancer monitoring of colorectal cancer patients."
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Lauschke et al., "Detection of APC and k-ras mutations in the serum of patients with colorectal cancer," *Cancer Detect Prev*.	Detection of K-ras and APC mutations in **serum DNA** of **colon** cancer patients.	Mutations in the K-ras oncogene only found in the serum of 6/22 patients but mutations in the APC tumor suppressor found in serum of 20/25 patients

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25(1):55-61 (2001).		
Schott et al., "Isolated tumor cells are frequently detectable in the peritoneal cavity of gastric and colorectal cancer patients and serve as a new prognostic marker," *Ann Surg.* 227(3):372-9 (1998).	**Bone marrow** or **peritoneal cavity fluid** of **gastric** or **colorectal** patients were investigated immunocytochemically.	Little prognostic significance of positive bone marrow results but high correlation of results from peritoneal cavity fluid and survival rate.
Kersting et al., "Differential frequencies of p16(INK4a) promoter hypermethylation, p53 mutation, and K-ras mutation in exfoliative material mark the development of lung cancer in symptomatic chronic smokers," *J Clin Oncol.* 18(18):3221-9 (2000).	**Sputum** analysis in **lung cancer** patients for the oncogene K-ras and the tumor suppressors p53 and p16.	Differences on DNA level could be detected in 14-51% of patients if the markers were analyzed individually but in 69% if the markers were combined.
Eisenberger et al., "Diagnosis of renal cancer by molecular urinalysis," *J Natl Cancer Inst.* 91(23):2028-32 (1999).	Microsatellite alterations in **urine** and **serum** of **renal cancer** patients.	Alterations could be found in 76% of urine samples and 60% of serum samples.
Vogel et al., "Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor," *Clin Cancer Res.* 5(3):593-9 (1999).	Immunocytochemical analysis of **peritoneal cavity fluid** and **bone marrow** in **pancreatic cancer** patients.	In 39 and 38% of patients alterations could be found in peritoneal lavage or bone marrow, respectively. Combination of the two bodily fluids resulted in 52% of correctly diagnosed patients.
Weitz et al.,"Detection of disseminated colorectal cancer cells in lymph nodes, blood and bone marrow," *Clin Cancer Res.* 5(7):1830-6 (1999).	Expression analysis of CK by RT-PCR on **lymph nodes** (lymphatic system), **blood** and **bone marrow** samples of **colon cancer** patients.	CK detection in lymph nodes is of prognostic relevance.

Ref

Chang et al., "Molecular diagnosis of primary liver cancer by microsatellite DNA analysis in the serum," *Br J Cancer*. 87(12):1449-53 (2002).	Analysis for LOH in **serum** of **liver cancer** patients.	LOH found in 76.2% of patients.
Hickey et al., "Molecular detection of tumour DNA in serum and peritoneal fluid from ovarian cancer patients," *Br J Cancer* 80(11):1803-8 (1999).	Analysis of genetic alterations in **serum** and **peritoneal fluid** of **ovarian cancer** patients.	In 17/20 serum samples and 12/19 peritoneal fluid samples, genetic alterations could be detected.
A. Vogel et al., "Disseminated tumour cells. Their detection and significance for prognosis of gastrointestinal and pancreatic carcinomas". *Virchows Arch.* 439(2):109-17 (2001).	Review of the various **methods to detect disseminated tumor cells** used at that time and the prognostic relevance of the results gained from these studies.	"Our evaluation of the studies on colorectal, gastric and pancreatic ductal carcinomas indicates that detection of disseminated tumour cells in different compartments may lead to more accurate tumour staging."

3. These publications show that analysis of bodily fluids for the diagnosis of non-haematopoetic tumors was well accepted in the art at the time of applicants' invention because disseminated tumor cells or DNA of non-haematopoetic tumor cells can be found in nearly all bodily fluids (e.g., serum, saliva, bone marrow, peritoneal cavity fluid, sputum, urine, lymph nodes, and blood, as noted in the above publications). While negative results exist, they depend upon the marker/antigen/genetic alteration that is investigated. A striking example of this is the low significance in diagnosing colorectal cancer by the detection of mutations of the oncogene K-ras in comparison to mutation in the tumor suppressor genes APC or *p53* (see Lauschke et al. and Hibi et al.). However, both extra- and intracellular DNA have successfully been detected and analyzed in relation to tumor diagnoses of various cancers (see, e.g., Hibi et al., p. 1405, 1st column, which notes that "tumor DNA is released into circulation and is enriched in plasma and serum.").

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4. In conclusion, tumor diagnosis by the analysis of bodily fluids is well accepted and state of the art. Low performance of certain methods is due to poor marker quality and/or tool selectivity (e.g. antibodies) rather than to the method as such. The present invention helps to solve this problem because it introduces a new tumor marker which is appreciably downregulated in certain tumors. In addition, in contrast to many other tumor markers, such as PSA, the biological function of Elp as growth suppressor has been shown experimentally (in the disclosure of the specification). Consequently, Elp has already undergone validation as a diagnostic tumor marker. Therefore, the present application establishes the status of Elp as a tumor growth suppressor, and the state of the art at the time of filing establishes the knowledge with respect to the ability to detect tumors in tissues and body fluids by the detection of such markers.

5. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001.

Dated: August 8, 2006

Barbara Froesch

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APPENDIX LIST

Appendix A - Hibi et al., "Molecular Detection of Genetic Altemations in the Serum of Colofectal Cancer Patients," *Cancer Research* 58: 1405-1407 (1998)

Appendix B - El-Naggar et al., "Genetic heterogeneity in saliva from patients with oral squamous carcinomas:implications in molecular diagnosis and screening," *J Mol Diagn.* 3(4):164-70 (2001).

Appendix C - Spafford et al.," Detection of head and neck squamous cell carcinoma among exfoliated oral mucosal cells by microsatellite analysis," *Clin Cancer Res.* 7(3):607-12 (2001).

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Appendix E - Schott et al., "Isolated tumor cells are frequently detectable in the peritoneal cavity of gastric and colorectal cancer patients and serve as a new prognostic marker," *Ann Surg.* 227(3):372-9 (1998).

Appendix F - Kersting et al., "Differential frequencies of p16(INK4a) promoter hypermethylation, p53 mutation, and K-ras mutation in exfoliative material mark the development of lung cancer in symptomatic chronic smokers," *J Clin Oncol.* 18(18):3221-9 (2000).

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Appendix L - Vogel et al., "Disseminated tumour cells. Their detection and significance for prognosis of gastrointestinal and pancreatic carcinomas". *Virchows Arch.* 439(2):109-17 (2001).

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APPENDIX A

RESEARCH 58, 1405–1407, April 1, 1998]

ɜcular Detection of Genetic Alterations in the Serum of Colorectal cer Patients[1]

Hibi, C. Rahj Robinson, Susan Booker, Li Wu, Stanley R. Hamilton, David Sidransky, and Jin Jen[2]

ɜnt of Otolaryngology-Head and Neck Surgery, Division of Head and Neck Cancer Research [K. H., L. W., D. S., J. J.], Departments of Oncology [S. B., S. R. H., D. S., ! Pathology [C. R. R., S. R. H.], Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2196

ct

ıave searched for the presence of genetic alterations in serum DNA ɜ from 44 colorectal cancer patients. Microsatellite analysis using ɔolymorphic markers revealed loss of heterozygosity and/or microsat- stability in 35 of 44 (80%) primary tumors. No alterations were l in the paired serum DNA. We next used an oligonucleotide-mediated ch ligation assay to detect tumor specific gene mutations in the serum. the 16 cases with a K-*ras* gene mutation in the tumor, the same ɔn was detected in three paired serum samples. In the 10 cases with a ıtation in the tumor, the identical mutation was detected in seven onding serum samples. Comparison of the molecular analysis with diagnosis of these patients revealed that none of the seven Dukes' ; patients with a K-*ras* mutation in their tumors demonstrated a ɔn in the serum. In contrast, five of seven stage B patients with a *p53* ɔn in the tumor demonstrated a mutation in the paired serum 01, Fisher's exact test). Taken together, either a K-*ras* or *p53* mutation ɛcted in the serum in 40% of the 25 patients (95% confidence interval, %), whose primary tumors contained a mutation and in 23% of the 44 s (95% confidence interval, 12–38%) with colorectal cancer. The ıt detection of *p53* mutation in the serum of patients with early stage suggests a possible use of this approach for clinical prognosis and monitoring of colorectal cancer patients.

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ɔrectal cancer is one of the most common malignancies in the and can usually be cured if diagnosed at an early stage. Methods ɛct surgically resectable tumors could significantly reduce from this disease. Several genetic changes, such as the activa- f the K-*ras* oncogene, inactivation of *APC*, *p53* genes, and loss er tumor suppressor genes on chromosome 18q are involved in thogenesis of colorectal cancer. Assays based on the molecular ion of these genetic changes have been shown as potential ɔstic tools for colorectal cancer (1–4). Furthermore, the identi- ɔn of these genetic changes at sites away from the primary tumor ıelp to assess the extent of disease and overall tumor burden at diagnosis (5).

vious studies have proposed that tumor DNA is released into the ation and is enriched in plasma and serum (6, 7). Radioimmu- ays revealed that the serum of cancer patients contained approx- ly four times the amount of free DNA compared with normal ɔl (8). Based on these observations, studies have shown that it is ɔle to detect tumor-specific DNA in the serum of head and neck r patients and in the plasma of lung cancer patients using satellite analysis (9, 10).

In the present study, we have examined 44 colorectal cancer patients using microsatellite analysis and a more sensitive mutation-specific mismatch ligation assay for genetic alterations in primary tumors and the paired serum samples. In the 25 cases with K-*ras* and/or *p53* gene mutations in the tumor, 10 cases exhibited the same alteration in the matched serum DNA using the mismatch ligation assay. Additionally, early stage tumors with *p53* mutations appeared to be more likely to demonstrate the identical mutation in the paired serum sample than those with a K-*ras* mutation. These results indicate that genetic alterations present in the tumors of colorectal cancer patients can be detected in the serum of the same patient, and that this approach can be potentially used for cancer prognosis and cancer monitoring for this deadly disease.

Materials and Methods

Sample Collection and DNA Preparation. Forty-four primary tumors and corresponding normal tissues were prepared from microdissected sections of formalin-fixed, paraffin-embedded tissues obtained from surgical resection of colorectal cancer patients. All tumor specimens contained more than 70% neoplastic cells. The pathological stagings at surgery for patients with either a K-*ras* or *p53* mutation are shown in Table 1. Serum samples were collected from the same patients prior to tumor resection and stored at −80°C. Normal, tumor, and serum DNA were prepared as described previously (11).

Microsatellite Analysis. Dinucleotide markers used for microsatellite analysis were *D18S55*, *D18S58*, *D18S61*, and *D18S69* on 18q (12), *CHRNB1* and *D17S786* on 17p (8), and *D8S133* and *D8S254* on 8p (13). These markers were chosen because they are highly polymorphic and are located at regions frequently lost in colorectal cancer. One of the two PCR primers for each marker was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. PCR amplification was performed as described (12) using paired normal, tumor, and serum DNA samples. The amplified products were separated in 8% denaturing urea-polyacrylamide-formamide gels followed by autoradiography (14). LOH[3] was scored if the ratio of one allele was significantly decreased (>30%) in tumor or serum DNA compared with normal DNA by independent visual observation of at least two investigators. Microsatellite instability was identified when novel band(s) were present in the tumor sample in addition to the constitutive allele(s) observed in the normal control.

Mutation Analysis for the *p53* Gene. The PCR amplification of tumor DNA samples consisted of 40 cycles of 95°C for 30 s, 58°C for 1 min, and 70°C for 1 min for exons 5, 6, and 8, or 95°C for 30 s, 63°C for 1 min, and 70°C for 1 min for exon 7. The primers used were: exon 5, 5′-AGGAATTCACTTGTGCCCTGACTT and 5′-GAGGAATCAGAGGCCTGGG; exon 6, 5′-TGCCCCAGGCCTCTGATTC and 5′-CATCGAATTCCACTGACAACCACCCTT; exon 7, 5′-TGCTTGCCACAGGTCTCC and 5′-ATCGGTAAGAGGTGGGCC; and exon 8, 5′-GGACAGGTAGACCTGATTTCC and 5′-CCTCCACCGCTTCTTGTC. All PCR products were purified and sequenced directly using the AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA). The sequencing primers were 5′-AACCAGCCCTGTCGTCTC, 5′-GAGACCCAGTTGCAAACCA, 5′-GAGGCAAGCAGAGGCTGG, 5′-AGTGGTAATCTACTGGGA, and 5′-ACCTCGCTTAGTGCTCCCTG for exons 5–8, respectively.

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[2] ı whom requests for reprints should be addressed, at Johns Hopkins University of Medicine, 824 Ross Research Building, 720 Rutland Avenue, Baltimore, MD 2196.

[3] The abbreviation used is: LOH, loss of heterozygosity.

le 1 *K-ras and p53 gene mutations in the tumors and serum of colorectal cancer patients*

se no.[a]	Tumor stage[b]	Gene alteration	Amino acid substitution	Mutation in serum
1	D	*K-ras*	Gly 12 Val	−
4	D	*K-ras*	Gly 12 Asp	+
7	B	*K-ras*	Gly 12 Asp	−
11	B	*K-ras*	Gly 12 Cys	−
15	B	*K-ras*	Gly 12 Ala	−
17	B	*K-ras*	Gly 12 Ala	−
19	C	*K-ras*	Gly 12 Ala	−
25	C	*K-ras*	Gly 12 Val	−
27	C	*K-ras*	Gly 12 Asp	−
29	C	*K-ras*	Gly 12 Asp	−
31	B	*K-ras*	Gly 12 Asp	−
37	B	*K-ras*	Gly 12 Asp	−
40	B	*K-ras*	Gly 12 Asp	−
41	C	*K-ras*	Gly 12 Val	−
45	C	*K-ras*	Gly 12 Ser	+
46	C	*K-ras*	Gly 12 Val	+
5	C	*p53*	Arg 248 Gln	−
6	B	*p53*	g to a (intron 5)[c]	+
15	B	*p53*	Arg 306 Term	−
16	B	*p53*	Arg 273 Cys	+
20	B	*p53*	Val 272 Met	−
21	C	*p53*	250-253 del	+
28	B	*p53*	Asn 200 Ser	+
33	B	*p53*	Arg 306 Term	+
34	C	*p53*	t to c (intron 6)[c]	+
44	B	*p53*	176 del	+

ᴬll cases are listed by the mutations detected. The first 16 cases had K-*ras* gene ion, whereas the latter 10 had *p53* mutation.
Dukes classification.
These mutations are located at the indicated introns.

:ismatch Ligation Assay. All possible alterations at K-*ras* codon posi- 12a, 12b, and 13b were determined using a modified allele-specific :on assay (15). The first exon of K-*ras* was amplified as described (4) and as the template for three separate ligation assays (12a, 12b, and 13b) in .mor samples. For each ligation assay, 50 ng of PCR product were mixed 8 ng each of three mutation specific oligomers, 100 ng of blocking mer, and 8 ng of a common ^{32}P-labeled oligomer in a 20-μl reaction iining 150 mM NaCl, 10 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.5), 1 mM nidine, 1 mM DTT, 1 mM ATP, and 3 μg of T4 gene 32 protein hringer Mannheim). This mixture was denatured at 95°C for 5 min and 'ed to cool at room temperature for 15 min; at which time 1 unit of T4 : was added. The ligation was carried out at 37°C for 1 h and terminated :at inactivation at 68°C for 10 min. The [^{32}P]phosphate on the unligated gomer was removed by the addition of 1 unit of alkaline phosphatase and :quent incubation at 37°C for 30 min. The ligation products were sepa- on 12% denaturing polyacrylamide gel. The presence and the nature of :ions were determined based on the relative migration of the ligation icts formed in control experiments using templates with known K-*ras* :ions. Oligonucleotide sequences used for the ligation assays were de- :d previously (15).

e mutation-specific oligomers for the *p53* gene were designed individu- iccording to each *p53* mutation and used in the ligation assay. For ple, tumor 5 had a mutation in codon 248 (5'-CATGAACCGGAGGC- T to 5'-CATGAACCAGAGGCCCAT). To detect this alteration in the ı, the mutation-specific oligomer (5'-CATGAACCA), the adjacent ^{32}P- :d oligomer (5'-GAGGCCCAT), and the blocking oligomer (5'- ACGGAGGC) were synthesized, and the mismatch ligation assay was :med exactly as described for the K-*ras* gene mutations. When a serum e was found positive, the DNA extraction and PCR reaction were :ed at least once from the original serum sample to confirm the presence : mutant allele. In all cases, the presence and the percentage of mutant in the serum remained unchanged.

lts and Discussion

: tested the serum of 44 colorectal cancer patients for the pres- of various types of genetic alterations. We first examined the : status of 18q, 17p, and 8p in the primary tumor samples :se of their frequent loss in colorectal cancers. Subsequently, DNA from paired serum samples were examined to determine whether tumor-specific LOH or instability was present in the paired serum of these patients. Although LOH or a microsatellite shift of at least one locus was observed in 35 of 44 (80%) primary tumors, no loss of heterozygosity or microsatellite shifts were detected in the serum (Fig. 1). This negative outcome could be due to the following two reasons: (*a*) colorectal tumor DNA in the serum would be filtered by the liver and diminished or diluted in peripheral blood, because colorectal blood circulation passes through the portal vein before entering into the peripheral circulation; and (*b*) microsatellite analysis may not have enough sensitivity to detect small amounts of tumor DNA in the serum of a colorectal cancer patient (16).

To examine this second possibility, we proceeded with a more sensitive method to detect K-*ras* and *p53* gene mutations in the serum DNA. K-*ras* gene mutations are observed in about 50% of primary colorectal tumors of all stages and represent an ideal target for such an approach. *p53* mutations occur in up to 70% of advanced colorectal cancers and have been reported as a poor prognostic factor (17). K-*ras* status in all 44 tumors was determined using the ligation assay. Of the 16 cases having K-*ras* gene mutations in their tumors, the ligation assay was repeated using the paired tumor and serum samples, and the same mutation was observed in the serum in 3 cases (19%; Fig. 2*A*). Thirty-three tumors with sufficient DNA were sequenced for *p53* mutations in exons 5–8 of the gene. Of the 10 cases having a *p53* mutation in the tumor, the same mutation was found in the serum of 7 patients (Fig. 2*B*). In total, we identified either a K-*ras* or *p53* mutation in the serum in 10 of 25 patients whose tumor had a mutation. All alterations observed in the serum were confirmed at least once using independently extracted serum DNA samples. Furthermore, in the case of K-*ras*, although oligomers for all three possible mutations at a given codon position (*i.e.*, 12a, 12b, or 13b) were present in the same ligation reaction, only the mutation identical to the one present in the primary tumor was observed.

After completion of the mismatch ligation assay in all specimens, clinical-pathological data were correlated with the molecular analysis. The three patients with K-*ras* gene mutation in the serum had either Dukes' C or D disease. None of the seven stage B patients with a K-*ras* mutation in the tumor had a detectable mutation in the serum. In contrast, five of seven stage B patients with *p53* mutations demonstrated the identical *p53* mutation in the serum. In our study, only case 15 had both K-*ras* and *p53* gene alterations in the same tumor, but neither of these changes was detected in the serum. The results of the mutation-specific ligation assay for all 25 patients with a K-*ras* or *p53* mutation in the tumor are summarized in Table 1.

Recent evidence suggests that microsatellite shifts (low level instability) or LOH can be detected in the circulating tumor DNA from the serum

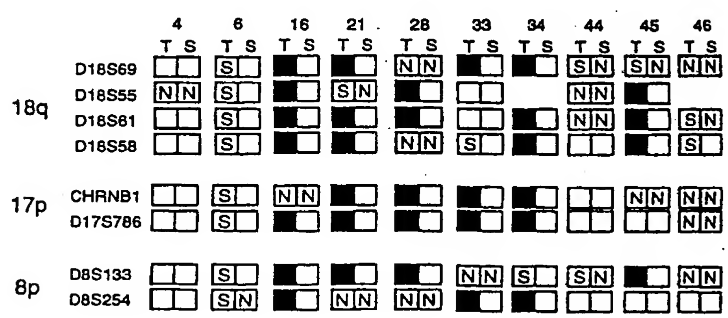
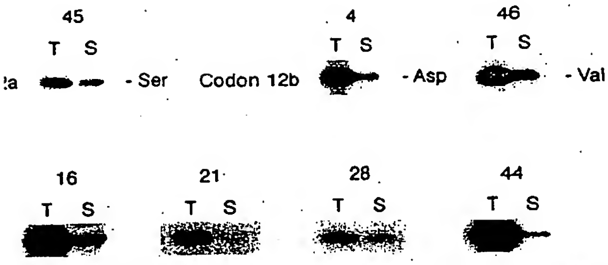


Fig. 1. Microsatellite analysis of paired tumor and serum samples. DNA was extracted from the tumor (*T*) and paired serum (*S*) of 44 colorectal cancer patients and analyzed as described in "Materials and Methods." Case numbers are indicated at the *top*. *Left*, microsatellite markers and their chromosomal location. *Open box*, retention; *closed box*, LOH; *S*, microsatellite shift (instability); *N*, not informative.



epresentative autoradiographs of the mismatch ligation assay. Examples of .ys detecting K-*ras* (*A*) and *p53* (*B*) mutations in the paired serum (*S*) and NA samples from patients with colorectal cancers.

2 *Genetic markers for the detection of colorectal tumor in the serum*

:tic alteration	Occurrence in tumor	Serum positive (%)
	31/44 (70%)	0/31 (0%)
:ellite instability	15/44 (34%)	0/15 (0%)
utation	16/44 (36%)	3/16 (19%)
ation	10/33 (33%)[a]	7/10 (70%)[b]

:nt DNA samples were available for complete sequence analysis in 33 cases

)14. Comparison of K-*ras* mutation *versus p53* mutation serum-positive cases exact test.

ıd neck cancer patients, and the plasma of small cell lung cancer), 10). Using reverse transcription-PCR, it has also been shown :tion of circulating tumor cells from the blood of colorectal .itients may have prognostic value or be related to stage at (18, 19). In this study, we tested four different genetic markers tection of colorectal cancer in the DNA from serum (Table 2). LOH and microsatellite shifts could not be detected, K-*ras* or :tions were detected in the serum of 40% of patients with a in the tumor using the mismatch ligation assay. Interestingly, ı-positive rate for tumors with a *p53* mutation was significantly ın that with a K-*ras* mutation ($P = 0.014$ by Fisher's exact test, Most of this difference can be explained by the *p53* mutant /hich displayed mutant serum DNA in patients with early stage t is conceivable that colorectal tumors with *p53* mutations may eased access to the peripheral blood due to the loss of the *p53*-induced apoptosis pathway. This result coincides with a ıt colorectal tumors with *p53* mutation are also more likely to ze (3).

:sults have two potential clinical applications: (*a*) patients at for colorectal cancer could be screened for the presence of lls in the serum by analysis of K-*ras* or *p53* gene mutations. n the method described in this study, such screenings are ›y the large number of different mutations identified in the :; and (*b*) a second application would be in the follow-up of liagnosed of colorectal cancers. In such patients, mutations of ;ene could first be identified using GeneChip technology[4] and d as targets for the mismatch ligation assay.

:ctal cancer is among the most common and fatal cancers in d. Thus far, the only noninvasive test for this disease is the f fecal occult blood. However, the appearance of hemoglobin is not specific for neoplasia. It has been shown that DNA :s from colorectal tumor cells could be amplified from the ing PCR for the detection of K-*ras* mutations (4), but it difficult to reproducibly PCR amplify tumor DNA from the this preliminary study, we detected tumor-associated DNA alterations in the serum of 23% colorectal cancer patients (10 of 44) by using either K-*ras* or *p53* mutation as a target. The clinical sensitivity of this assay can be potentially improved by incorporating other common genetic targets such as *APC*. Advances in technologies to permit rapid detection of an array of specific mutations would enhance the utility of this approach. Previous studies in cancer patients have suggested that those with genetic alterations in serum or plasma are more likely to develop metastases and die of their disease (9, 18). Further studies are needed to determine the clinical relevance of identifying specific genetic alterations in the serum of colorectal cancer patients for the prognosis and monitoring of the disease.

Ahrendt, personal communication.

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APPENDIX B

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Genetic Heterogeneity in Saliva from Patients with Oral Squamous Carcinomas

Implications in Molecular Diagnosis and Screening

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We performed microsatellite analysis at chromosomal regions frequently altered in head and neck squamous carcinoma on matched saliva and tumor samples from 37 patients who had oral squamous carcinoma. The results were correlated with the cytologic findings and traditional clinicopathologic factors to assess the diagnostic and biological potential of these markers. Our data showed that 18 (49%) of the saliva samples and 32 (86%) of the tumors had loss of heterozygosity (LOH) in at least one of the 25 markers studied. In saliva, the combination of markers D3S1234, D9S156, and D17S799 identified 13 (72.2%) of the 18 patients with LOH in saliva ($P <$ 0.001). For tumors, markers D3S1234, D8S254, and D9S171 together identified 27 (84.3%) of the 32 tumors with LOH at any of the loci tested ($P < 0.001$). Eleven (55%) of the 20 saliva samples with cytologic atypia and seven (35%) of the 17 specimens without atypia had LOH. Significant correlation between LOH in tumor at certain markers and smoking and alcohol use was found. Our results indicate that: 1) epithelial cells in saliva from patients with head and neck squamous tumorigenesis provide suitable material for genetic analysis; 2) combined application of certain markers improves the detection of genetic alteration in these patients; 3) clonal heterogeneity between saliva and matching tumor supports genetic instability of the mucosal field in some of these patients; and 4) LOH at certain chromosomal loci appears to be associated with smoking and alcohol consumption. *(J Mol Diag 2001, 3:164–170)*

The oral cavity is an ideal site for screening individuals at high risk of developing head and neck squamous carcinoma (HNSC) because of the availability of cells shed in saliva and the convenience of visualizing and sampling lesions at these locations.[1–3] However, little progress has been made in the management of patients with oral squamous carcinoma due mainly to the nonspecific symptoms, minimal physical finding inpatients with early-stage cancer, and the lack of biological predictors of progression.[4] Identifying novel and reliable biogenetic markers for the biological assessment of squamous lesions may assist in early diagnosis and treatment of head and neck squamous tumorigenesis. Microsatellite DNA motifs consisting of highly polymorphic short tandem repeat sequences distributed throughout the genome have been widely and successfully used as markers for molecular analysis of tumorigenesis in head and neck and other neoplasms.[1,5–7]

Studies using microsatellite markers from different chromosomal arms in HNSC have shown that alterations at certain regions on chromosomes 3p, 9p, 17p and 18q to be associated with the development of these tumors.[1,2,6,8–12] Although the timing and the order of these alterations are currently unknown, different studies have shown high incidence of loss of heterozygosity (LOH) in noninvasive lesions indicating an early association with tumorigenesis.[1,6,7] Analysis of selected microsatellite markers at these regions on epithelial cells from patients' saliva is a convenient and non-invasive approach for molecular screening and early detection of this disease.[13–24] In this study, we evaluated the diagnostic and biological implications of the alterations at several of the above mentioned microsatellite markers in prospectively collected saliva and tumor specimens from patients who had oral squamous cell carcinoma.

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Materials and Methods

Forty matched histologically normal squamous mucosa and tumor tissues and saliva collected freshly from patients with primary untreated HNSC were prospectively acquired between 1996 and 1998 by the Department of Pathology at The University of Texas M. D. Anderson Cancer Center after obtaining patient consent. Ten saliva samples from normal individuals with negative history of cancer were also collected and used as a biological control. Histologically normal squamous mucosa from each cancer specimen was stripped from the farthest margin after frozen section evaluation and used as putative controls. Ficoll-Hypaque isolated lymphocytes from heparinized peripheral blood from all patients and 10 normal volunteer individuals were also obtained and immediately frozen; lymphocytes were used as control for saliva analysis from normal volunteers. Lymphocytes were used only if histologically matched mucosa was suspected to harbor alterations.

For all patients, tobacco and alcohol consumption histories were obtained from the epidemiology database. Patients' demographic, pathological, and clinical information was collected retrospectively from pathology reports and patient records. The 10 normal saliva samples from healthy individuals were obtained from five nonsmokers and five current smokers.

Saliva Collection

After the mouth of each patient and normal subject was washed with sterile water, saliva specimens were collected in a sterile cap and transported immediately to the laboratory to be centrifuged at 1200 g for 5 minutes. The supernatants were decanted and the cell pellets were frozen at −80° in the same tubes. Three cases were eliminated for lack of sufficient DNA from saliva samples and the remaining 37 cases formed the cohort of materials for the analysis.

DNA Extraction

Extraction of DNA was performed using DNAzol (Molecular Research Center, Cincinnati, OH). Cells were lysed in DNAzol using a Tissue Tearor (Biospec Products, Bartlesville, OK) for fresh tissue, and a vortex homogenizer for saliva. The liberated DNA was precipitated with ethanol and resuspended in 10 mmol/L Tris, 1 mmol/L EDTA.

Microsatellite Analysis

Aliquots of DNA were subjected to standard polymerase chain reaction (PCR) analysis using primers for the following loci: D3S656, D3S1293, D3S1234, D3S1217, D3S1261, D8S254, D8S261, LPL-tet, D8S298, D8S283, D9S104, D9S156, D9S168, D9S171, D9S199, D17S513, TP53, D17S799, CHRNB1, D17S122, D18S46, D18S363, D18S35, D18S39, and D18S41 (Research Genetics, Huntsville, AL). The loci were chosen based on the frequency of their alteration in head and neck tumors. Microsatellite location on the chromosomal arms was determined based on the latest map in the Genetic Location Database of the University of Southampton, U. K.

One primer was end-labeled using γ^{32}P-ATP and T4 polynucleotide kinase. PCR was performed in a 20 μl volume with 10 ng of genomic DNA, 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.001% gelatin, 0.5 μmol/L of each unlabeled primer, 0.01 μmol/L labeled primer, 0.2 mmol/L dNTPs, 5% dimethyl sulfoxide (Sigma Chemical, St. Louis, MO), and 0.5 units of Amplitaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT).

The amplification process consisted of: (a) an initial 10-minute denaturation step at 94°; (b) 35 cycles of denaturation at 94° for 30 seconds, annealing at 55–60° for 1 minute, and elongation at 72° for 1 minute; and (c) a final elongation at 72° for 5 minutes. Sequencing stop buffer was added to the reactions. PCR products were then denatured at 94° for 5 minutes and quickly chilled, and 4–6 μl was loaded on 7% acrylamide-urea sequencing gels containing 32% formamide. Electrophoresis was performed at 80 W for 2–4 hours, depending on the fragment size. The gels were dried and exposed to Hyperfilm-MP (Amersham, Arlington Heights, IL).

Evaluations were conducted by two independent observers who visually scored the pattern and the band intensity between normal tissue, tumor, and saliva specimens from each case before the identification of the patients. LOH was defined by the presence of an allelic band difference between nonmalignant epithelium and tumor or saliva of more than 50%. Visually suspicious cases were subjected to densitometry, and a difference of >30% was scored as LOH. Instability was defined as the appearance of a novel band that was not seen in the normal control.

Acridine Orange Flow Cytometry

Disaggregated cells were adjusted to 1.0×10^6 cells/ml. A cytospin preparation was evaluated for quality and cellular integrity. Cells were subsequently stained with acridine orange according to the two-step procedure of Traganos et al.[25] Ploidy status was defined by the DNA index, which represents the ratio of the relative G_0/G_1 stemline portion of the tumor samples to that of the normal peripheral blood lymphocytes. Diploid DNA is defined by a single G_0/G_1 peak with a DNA index of 1.0, and DNA aneuploidy is defined by the presence of one or more additional stemlines to the right (hyperdiploid, DI > 1.0) or the left (hypodiploid, <1.0) of the G_0/G_1 diploid peak. A near diploid (hypodiploid or hyperdiploid) DI was determined after mixing the test sample with lymphocyte controls. The coefficient of variation (CV) of DNA diploid and aneuploid stemline ranged from 2.1 to 5.4 with a mean of 3.6 ± 1.2 and 2.8 to 6.1 with a mean of 4.9 ± 1.6, respectively.

Statistical Methods

All correlations were performed using a two-tailed Fisher's exact test.

Table 1. Clinicopathological and Epidemiological Characteristics of Patients with Oral Squamous Carcinoma

Case no.*	Sex	Grade	Site	Size (cm)	Stage	Sm†	Alcohol HX	DI	PI
1	M	MD	Tongue	4.9	II	Yes	Yes	1	7
2	F	PD	FOM	n/a	U	No	No	1	10
3	M	MD	Palate	4	I	Yes	Yes	1	13
4	M	WD	Tongue	2.5	II	Quit	Yes	1.67	6
5	M	WD	Tongue	2.5	II	Quit	Quit	1.82	9
6	F	PD	Palate	5.5	U	Yes	None	1	3
7	M	MD	Tongue	2	II	Quit	None	1.82	17
8	F	MD	Tongue	0.9	III	Yes	Social	1.9	8
9	M	MD	Tongue	4	III	Yes	Social	1	5
11	M	MD	Tongue	n/a	III	Yes	Social	1	13
12	M	MD	Tongue	4.6	III	Yes	Quit	1	19
13	M	MD	FOM	2.2	III	Yes	Yes	1.71	27
14	M	MD	Tongue	4.5	II	Yes	Yes	1	22
15	M	MD	Tongue	1.5	U	Unknown	Social	1.35	17
16	F	WD	FOM	4.5	II	No	No	1	6
18	M	MD	Tongue	2	II	Yes	Yes	1.72	10
19	M	PD	Tongue	4.5	III	Yes	No	1	8
20	M	MD	Tongue	n/a	III	Yes	Quit	1.58	16
22	M	MD	FOM	4	U	Yes	Quit	n/a	n/a
23	M	PD	Tongue	1.7	U	Quit	Yes	1.73	24
24	M	PD	Tongue	0.7	I	Quit	Yes	n/a	n/a
25	M	PD	FOM	8	III	No	No	1	n/a
26	M	MD	Tongue	2	IV	No	Social	1	9
27	M	PD	FOM	n/a	III	Quit	Quit	n/a	n/a
28	M	MD	FOM	n/a	II	Yes	Yes	n/a	n/a
29	F	MD	FOM	n/a	III	Quit	Yes	n/a	n/a
30	F	MD	Tongue	n/a	II	No	Social	n/a	n/a
31	M	MD	Tongue	1.5	II	Yes	Social	n/a	n/a
32	M	MD	Tongue	1.8	III	Quit	Social	1	5
33	M	MD	Tongue	2.2	II	No	Social	1	14
34	F	MD	Tongue	n/a	II	No	No	n/a	n/a
35	M	PD	Gingiva	2.7	III	Quit	Unknown	1	4
36	F	MD	Tongue	1.5	II	Yes	Social	n/a	n/a
37	M	PD	Tongue	3.5	III	Yes	Quit	1.66	6
38	F	PD	Tongue	2	IV	Yes	Social	2.33	12
39	F	MD	Tongue	n/a	II	No	No	n/a	n/a
40	M	WD	FOM	n/a	I	Quit	Social	1	5

* Cases 10, 17, and 21 were dropped during the study for lack of sufficient DNA to complete the study.
† Quit: ceased smoking for at least one year and no current drinking; Yes: alcohol >7 drinks/week; smoking, at least 1 pack/day.
M, male; F, female; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; FOM, floor of mouth; U, unknown; DI, DNA index; PI, proliferative index; Sm, smoking; n/a, not available.

Results

Table 1 presents the clinicopathologic and epidemiological characteristics of the 37 study subjects. None of the histologically normal squamous mucosas used as a control showed any alterations at the microsatellite sites tested. Accordingly, no further analysis of the lymphocytes from these patients were performed. The incidence of LOH at individual markers and its occurrence per chromosomal arm for both tumor and corresponding saliva samples are presented in Table 2 and Figure 1. Chromosomes 9p, 3p, and 17p showed the highest incidence of LOH in both tumor and saliva. We also investigated the use of small combinations of markers to improve the detection of genetic alterations in both saliva and tumor samples as a likely approach for future clinical applications. Figure 2 illustrates LOH found in tumor and saliva (A), tumor alone (B), and saliva alone (C).

Saliva

Of all 37 saliva specimens, 18 samples (49%) manifested LOH, and 19 samples (51%) lacked any abnormalities. The most frequent LOH (21.6%) was found at marker D3S1234 (8 cases), but adding markers D17S799, and D9S156 (13 35%) of the 37 cases analyzed showed LOH which led to 100% specificity and 44% sensitivity of LOH detection. Eleven instances of instability were identified in saliva from three cases. None of the 10 informative saliva samples from healthy individuals showed any LOH at the markers used.

Tumor

Thirty-two (86%) tumors showed LOH in at least one marker, and five (14%) had no LOH. The two markers that exhibited LOH most frequently in tumor tissue were D9S171 and D9S156, which identified 17 (53%) inci-

Table 2. Incidence of LOH in Heterozygous Cases (%) of Saliva and Tumor Specimens of Patients with HNSC

Marker	Heterozygous (informative)	Tumor only	Saliva only	Tumor and Saliva
D3S656	24	10	0	2
D3S1293	32	5	2	2
D3S1234	23	7	3	5
D3S1217	33	11	2	2
D3S1261	33	12	2	1
D8S254	26	11	1	0
D8S261	23	9	0	0
LPL-tet	28	9	0	1
D8S298	26	7	0	0
D8S283	30	7	1	0
D9S104	31	12	1	1
D9S156	28	16	3	1
D9S168	23	11	2	1
D9S171	29	16	2	1
D9S199	32	13	0	1
D17S513	27	10	0	2
TP53	33	9	1	1
D17S799	29	9	1	2
CHRNB1	29	9	1	1
D17S122	26	7	0	1
D18S46	31	11	1	1
D18S363	20	7	0	0
D18S35	28	9	0	1
D18S39	22	10	0	0
D18S41	23	6	0	0

dences of LOH each. The combination of markers D9S171 + D3S1234 identified 24 (75%) of the 32 tumors with LOH ($P = 0.051$). Addition of marker D8S254 to the above combination identified three more instances of LOH for a total of 27 cases ($P = 0.009$). This marker combination gave 84% sensitivity and 100% specificity of LOH detection. Various other combinations of markers, especially those of D9S171, D3S1234, D8S254, and D9S156, identified similar incidence of tumors with LOH.

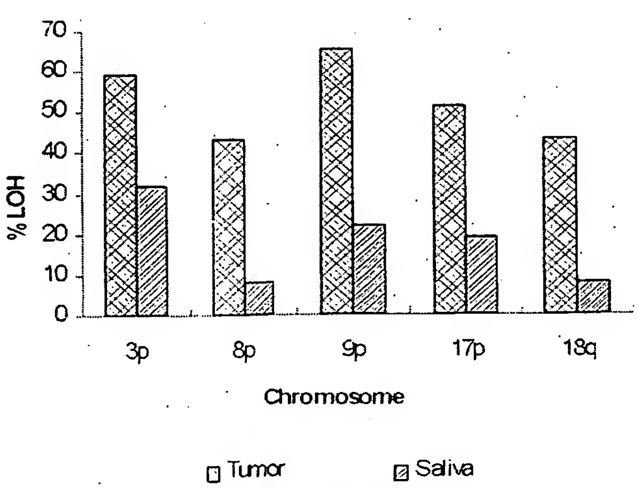

Figure 1. Frequency of LOH by chromosomal arm in tumor and saliva samples.

Four incidences of instability were also noted in tumors from four different patients.

Saliva versus *Tumor*

Sixteen tumors from the 18 patients with LOH in saliva samples (in one or more of the 25 markers) had LOH in corresponding tumors. Also, 16 tumors from the 19 patients with no LOH in saliva samples had LOH ($P = 1.00$, Fisher's exact test); the occurrence of LOH in saliva did not predict the LOH in corresponding tumor samples. One pair of markers (D3S1234 + D9S199) showed a statistically significant correlation in LOH between saliva and tumor ($P = 0.038$), as did many sets of three markers. The majority of these sets of three markers showed high agreement between saliva and tumor (LOH in both saliva and tumor or lack of LOH in both saliva and tumor). However, there was no significant correlation between LOH in saliva and in tumor for individual markers. In three instances, reciprocal LOH was noted between saliva and matching tumor specimens (Figure 2A). In these instances, the finding was considered discordant. No concomitant instability was found in any saliva and tumor samples manifesting this feature.

Cytologic Atypia and LOH in Saliva

No malignant cells were identified in any of the saliva samples by cytologic examination of giemsa-stained slide preparation. Twenty (54%) of the 37 saliva specimens had cytologic atypia. Nine (45%) of the 20 cases with cytologic atypia had no alterations, and 11 of the remaining (55%) had LOH; 7 (41%) of the 17 samples with cytologically normal epithelial cells had LOH in at least one marker ($P = 0.79$). Although no correlation between LOH at single marker and atypia was found, two markers showed the highest association with cytological atypia (D9S156 and D9S168, $P = 0.132$ and $P = 0.217$, respectively). Combining either of these markers with D3S1261 showed statistical correlation between LOH and atypia ($P = 0.02$) with 60% and 100% specificity ($P = 0.020$). The inclusion of a third marker did not improve the correlation.

LOH and Clinicopathological Factors

A correlation between certain marker combinations and smoking history at four markers alone or in various combinations (D3S1293, CHRNB1, D8S298, and D9S104) showed correlation with this feature. This and other quadruple combinations of markers identified LOH in 15 of the 18 smokers ($P = 0.0001$). Interestingly, patients who quit smoking showed an LOH frequency that was between that of smokers and nonsmokers: five of the 10 patients who quit smoking had LOH in at least one of these markers in their tumors. Multiple markers in tumors correlated with stage (e.g., D3S1261, D8S283, D9S156, D9S168, and TP53); the combination with the highest correlation was D3S1261, D9S156, and TP53 ($P = 0.002$). Similarly, the combination D18S363 and D8S283

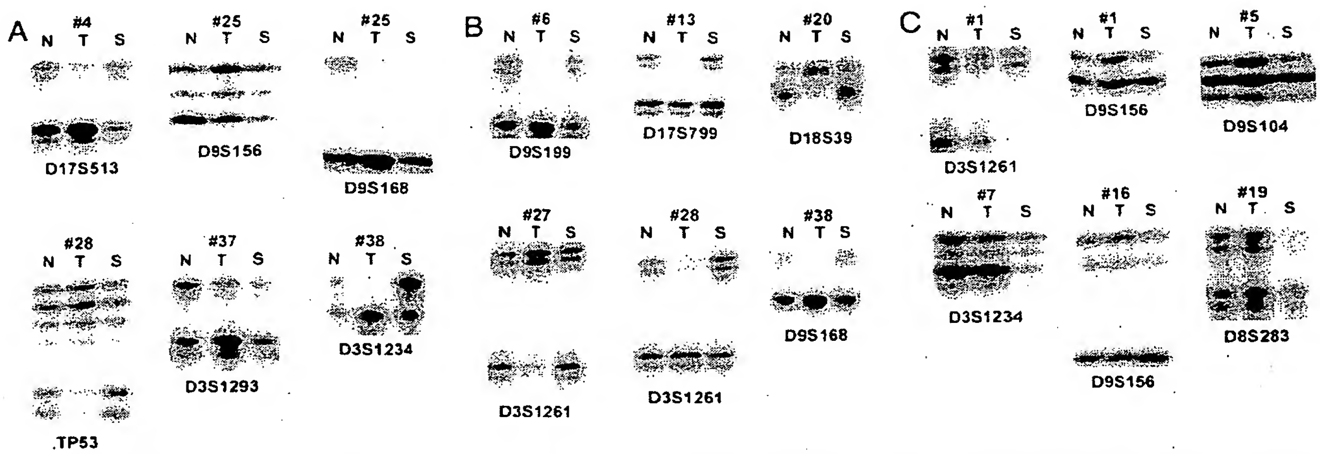

Figure 2. Representative illustrations of LOH in specimens. **A:** LOH in both tumor and saliva; **B:** LOH in tumor alone; **C:** LOH in saliva alone. N, normal; T, tumor; S, saliva.

was significantly correlated with the DNA index (P = 0.002). The only factor significantly correlated with LOH at marker D3S1248 in saliva was the DNA index (P = 0.014).

Saliva from Smoking and Nonsmoking Normal Volunteers

All of the 10 normal saliva samples were informative for at least one of the markers used. None of the DNA extracted from the 10 saliva specimens from normal nonsmoking and smoking individuals manifested any LOH at the markers analyzed. Only one saliva specimen from a normal smoker showed instability at one marker on the short arm of chromosome 3.

Discussion

Molecular genetic studies of HNSC have demonstrated frequent genetic alterations at certain chromosomal regions in premalignant lesions and invasive tumors.[1–9] The studies also showed that certain regions on chromosomes 3p, 9p, 8p, and 17p are frequently altered in dysplastic lesions[1,6–8] and may constitute an early event in lesion development. Analysis of these markers in oral secretions and other accessible specimens may allow for rapid, inexpensive, and objective assessment of the genetic abnormalities at these sites for early detection, screening of individuals at high risk, and follow-up of patients with cancer.[2,3,26]

In this study, approximately 50% of the saliva samples and 86% of the tumor specimens manifested microsatellite LOH. The incidence of LOH in saliva, however, could have been higher had a method to enrich the epithelial cells in specimens been used.[27] The results, however, support those of previous studies and further underscore the early association of these markers with HNSC tumorigenesis.[1,2,6,7] The genetic heterogeneity between saliva negative for malignant cells and corresponding tumor specimens in certain cases, highlight the presence of genetic alterations in the squamous epithelial cells lining the oral cavity exclusive of the cancer site.[28,29,30–34]

We, however, found no LOH in any of the histologically normal squamous mucosas used as control and we attribute this to the relatively small contribution of the squamous epithelium relative to the subepithelial elements in the tissues used. Microdissection of epithelial cells may have led to the identification of LOH in these histologically nondysplastic squamous mucosa as reported in at high risk patients[7,34] studied by our group. Nonetheless, the finding of LOH in saliva samples lacking malignant cells on cytologic evaluation, lends further credence to the field cancerization hypothesis and the increased risk of a second primary cancer developing in these patients[7,26,35,36,37]. Although none of the patients in this study manifested evidence of recurrence or secondary tumors thus far, a longer follow-up period is required to substantiate this notion. Other studies, however, have shown a high concordance in matched secretion and tumor specimens, indicating common clonal derivation[16,17,20,23,24,38,39]. In these studies, however, malignant cells were identified and analyzed in the cellular sources used.

In our study, although no cytological evidence of malignancy in any of the saliva samples was identified, cytologic atypia was found in more than 50% of the specimens. This feature correlated significantly with LOH at two markers on the short arm of chromosomes 3 and 9. Our findings, along with those from previous studies of head and neck, lung, and bladder tumors indicate that combining molecular and cytologic analyses in secretions may provide additional information for better identification of high risk patients[5,7–9,20,39,40]. Our results also show that alterations at microsatellite markers in tumors correlated significantly with aggressive clinicopathologic factors in these patients. A similar correlation has been reported in other studies, indicating that alterations in certain chromosomal loci are associated with the pathobiologic characteristics of these tumors and may be used for the biological assessment of these tumors.[10,11] Of particular interest in our study is the finding of a signifi-

cant correlation between LOH at certain chromosomal regions in tumor and smoking and alcohol consumption. Additional investigations of these regions may lead to identifying the markers or genes that are associated with carcinogenesis. Previous studies have also reported an association between LOH in these same chromosomal loci and tumors in the upper respiratory tract.[41,42]

In conclusion, the results of our study indicate that, in patients with HNSC, saliva is a readily available source of DNA for genetic analysis.[42,43] Enriching for epithelial cells may increase the correlation obtained between saliva and tumor specimens. Alternatively, cells obtained by buccal brushing of multiple sites in the oral cavity may be used as a noninvasive substitute for molecular analysis.[26,44] Our results lend molecular evidence for field cancerization[45,46] and the use of selective genetic markers in early detection of HNSC in people at high risk.[24,43,47]

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APPENDIX C

Detection of Head and Neck Squamous Cell Carcinoma among Exfoliated Oral Mucosal Cells by Microsatellite Analysis[1]

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ABSTRACT

Prompt detection of head and neck squamous cell carcinoma (HNSCC) is vital to successful patient management. In this feasibility study, we used microsatellite analysis to detect tumor-specific genetic alterations in exfoliated oral mucosal cell samples from patients with known cancer. Exfoliated mucosal cells in pretreatment oral rinse and swab samples were collected from 44 HNSCC patients and from 43 healthy control subjects (20 nonsmokers and 23 smokers). We tested a panel of 23 informative microsatellite markers to assay DNA from the matched lymphocyte, tumor (from cancer cases), and oral t est samples. Loss of heterozygosity or microsatellite instability of at least one marker was detected in 38 (86%) of 44 primary tumors. Identical alterations were found in the saliva samples in 35 of these 38 cases (92% of those with markers; 79% overall) including 12 of 13 cases with small primaries [stage T_1 or T_x (occult primary)] and 4 of 4 cases of patients that had undergone prior radiation. Microsatellite instability was detectable in the saliva in 24 (96%) of 25 cases in which it was present in the tumor, and loss of heterozygosity was identified in the test sample in 19 (61%) of 31 cases. No microsatellite alterations were detected in any of the samples from the healthy control subjects. This approach must now be refined and validated for the detection of clinically occult disease. Microsatellite analysis of oral samples may then become a valuable method for detecting and monitoring HNSCC.

INTRODUCTION

HNSCC[3] affects 50,000 Americans and >500,000 individuals worldwide each year (1). Early detection can improve patient survival and diminish the morbidity of treatment required for advanced disease. However, early detection is hindered by several factors: (*a*) risk factors (tobacco and alcohol use) and early warning signs (such as hoarseness and otalgia) are not universally understood; (*b*) patients often delay seeking medical attention for a variety of psychological and social reasons; (*c*) examination of the upper aerodigestive tract requires expertise and equipment not possessed by many clinicians; and (*d*) even when a provider has the necessary capability, a tumor may remain undetected because many head and neck cancers occur in hidden sites such as crypts in the base of tongue or tonsil, or beyond view in the hypopharynx and larynx. Failure to diagnose HNSCC in its earliest stages is perhaps the greatest factor contributing to the poor outcome for treatment of this disease. In the larynx, for example, stage I squamous cell carcinoma is successfully treated in 90% of cases. In contrast, less than 50% of stage IV tumors are controlled, often with loss of the larynx (2). Prompt detection is also vital to effective surveillance after treatment of HNSCC. The opportunity for successful surgical salvage after chemotherapy and/or radiation therapy is lost if the residual cancer grows or spreads while remaining hidden amid scar or inflammation. Beyond clinical examination, no early detection technique currently exists for squamous cell carcinoma of the upper aerodigestive tract. Innovations in early detection technology offer a means to reduce morbidity and mortality of HNSCC using current treatment options.

Microsatellite alterations have been used as markers of clonality (3) and to detect cancer cell DNA in a background of normal cells (4). Microsatellite analysis can reveal either LOH or MSI in the amplified microsatellite repeat locus. Tumor-specific DNA alterations can be found in the body cavity fluids and blood of patients with various cancers. For example, tumor-specific microsatellite alterations can be consistently detected in the urine of patients with bladder carcinomas (5) and can be detected in the serum of a significant percentage of patients with HNSCC (6). The purpose of his study was to determine whether tumor-specific microsatellite alterations may also be detectable in the DNA from exfoliated oral cells in saliva of HNSCC patients. We planned to assess a panel of microsatellite markers in a group of subjects with clinically apparent cancer and healthy control subjects as a first step in the development of a screening tool.

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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; LOH, loss of heterozygosity; MSI, microsatellite instability.

Table 1 Tumor characteristics

Tumors from 44 patients with HNSCC were analyzed at 23 microsatellite loci and results were compared with DNA from peripheral blood lymphocytes. Exfoliated oral mucosal cells were also analyzed with the same panel of microsatellite markers. The location of the tumors was primarily in the oral cavity and oropharynx, accessible to the rinse/swab harvesting of exfoliated cells. The T stage reflects the surface area of the tumors in general and is distributed from clinically inapparent (T_x) to extensive T_4 lesions.

	n	Marker in tumor	Marker in oral sample
Site			
Oral cavity	13	11	11
Oropharynx	22	18	15
Larynx	5	5	5
Hypopharynx	1	1	1
Unknown	3	3	3
T stage			
T_1	13	10	9
T_2	8	7	6
T_3	5	5	4
T_4	11	10	10
rT_4[a]	4	3	3
T_x	3	3	3

[a] rT_4, recurrent T_4.

MATERIALS AND METHODS

Patients

The study involved 44 patients with HNSCC and 43 healthy control subjects including 20 nonsmokers and 23 smokers. All of the smoking control subjects had at least a 20-pack-year smoking history and continued to smoke at the time of sample collection. The majority of cancer subjects had newly diagnosed HNSCC, although six patients were entered into the study at the time of diagnosis of a recurrent cancer or second primary cancer after previous radiation therapy. Each of the individuals in the control group underwent a physical examination by a head and neck surgeon, including inspection of the oral cavity, pharynx, and larynx to ensure that no suspicious mucosal lesion was present. An Institutional Review Board-approved informed consent was obtained from each participant. Table 1 provides the site and stage of the tumors included in the study.

Tissue Collection

All of the samples were collected by a head and neck surgeon (M. F. S., W. M. K.). Tumor and blood were obtained from each HNSCC patient at the time of biopsy or resection. In three cases, the tumor sample was taken from a metastatic lymph node of a patient who had presented with cancer arising in an unknown mucosal primary site (unknown primary). Fresh tumor tissue obtained at surgery was used when available. In six cases, the tumor was microdissected from paraffin-embedded tissue. Ten ml of blood were collected in citrated tubes as a source of normal control DNA. Patients contributed a pretreatment oral rinse by swishing and gargling for 15 s with 25 ml of sterile 0.9% NaCl. Then the oral cavity was swabbed by the surgeon using a cotton-tipped applicator. Three strokes were performed of each buccal surface, the alveolar ridges, lateral tongue, floor of mouth, and pharyngeal inlet (tonsil, soft palate, and posterior tongue). The applicator was rinsed in saline after stroking each region. At the beginning of the study, the rinse and swab material was combined immediately, and analyzed together. Later, the rinse and swab samples were kept separate, and analyzed individually. All of the results will be reported as combined samples because there was no significant advantage seen in the analysis of separate rinse and swab specimens. In six cases, the tumor involved a site (five larynx, one hypopharynx) that would not have been sampled under the protocol of the swabbing just described. The test sample in these cases was taken at the time of examination under anesthesia, and included a swab of the hypopharynx and supraglottic larynx. Blood and oral rinse-swab samples were also collected from the 43 control subjects. All of the oral samples were collected in a sterile, closed container, refrigerated immediately, and processed to collect DNA within 8 h.

DNA extraction

Tumor

Frozen Tissue. Representative thin tumor sections were stained with H&E. Microdissection was performed as needed, removing noncancerous tissue to achieve at least a 70% purity of the neoplastic cell population. Ten 12-μm tissue sections were placed in 1% SDS/proteinase K (0.5 mg/ml) and incubated at 48°C for 72 h.

Paraffin-embedded Tissue. Representive tumor samples were sectioned producing 25 14-μm samples. They were placed on glass slides and microdissected using a dissecting microscope. The samples were placed in xylene overnight for deparaffinization, pelleted in 70% ethanol, dried, and incubated in SDS/proteinase K at 48°C for 72 h.

Blood

Ten ml of blood were brought to 30 ml with TM buffer (2% Tris-EDTA-MgCl). Lymphocytes were collected by centrifugation at 2500 rpm for 15 min and were placed in 1% SDS/proteinase K (0.5 mg/ml) at 48°C for 72 h.

Exfoliative Oral Cells

The 25-ml rinse and swab (test) samples were subjected to centifugation at 2500 rpm for 15 min. The supernatant was discarded, and the cell pellet was retained and placed in 1% SDS/proteinase K (0.5 mg/ml) at 48°C for 72 h. Digested tissue and fluids from all of the sources were then subjected to phenol-chloroform extraction and ethanol precipitation.

Microsatellite Analysis of DNA

A panel of twenty-three tetranucleotide microsatellite repeat PCR primers (Research Genetics, Huntsville, AL) that had been used in similar studies in our laboratory were selected for this study (Table 2). Several of these alterations were known to be highly informative because of a study of the allelotype of HNSCC (6). Others were identified in studies of lung (7) and bladder carcinoma (8). This experience indicated a high frequency of MSI in microsatellites consisting of $(AAAG)_n$ repeats, and, therefore, the panel preferentially included those markers. Prior to amplification, 50 ng of one primer from each pair was end-labeled with [γ-^{32}P]ATP (20 mCi; Amersham Life

Table 2 Microsatellite panel

A panel of 23 microsatellite sequences were used in an effort to identify at least one tumor-specific marker for each of 44 HNSCC lesions. The panel includes markers from chromosomal loci known to have a high likelihood of allelic loss in HNSCC, and many $(AAAG)_n$ sequences. These tetramers were particularly useful with a high rate of MSI in the tumors.

Microsatellite	Location	Repeat sequence	No. of markers, T/S[b] LOH	MSI
D9S753	9q21.1–22.3	$(A)_5(AAAG)_{18}(AGAG)_4(A)_9$	1/1	6/4
D20S77	20	$(AAAG)_{21}$	0	2/1
UT5307	8	$(AAAG)_{19}$	1/0	1/1
D9S242	9q32–33	$(AAAG)_{26}(AAAG)_3$	1/0	1/1
CSFIR-6	5q33.4–34	(TAGA)	2/1	1/1
D11S488	11q24.1–25	$(AAAG)_{14}(GAAG)_{10}$	4/1	1/1
ACTBP2	5	$(AAAG)_{26}$	5/2	1/1
D8S321	8q24.13–8qter	$(AAAG)_{12}$	1/0	3/1
UT5320	8	$(AAAG)_{10}$	2/1	3/2
D9S171	9p21	$(A)_{10}$	2/2	3/2
D9S162	9p22–21	$(CA)_{24}$	2/2	2/1
D20S82	20	$(AAAG)_{10}$	1/0	1/0
D20S85	20	$(AAAG)_{12}$	1/1	0
Li7686	7q31–32	$(AAAG)_{30}(AAGG)_{22}$	0	5/4
FGA	4q28	$(TCTT)_{13}$	2/1	4/3
D9SIFNA	9p22	$(CA)_n$	7/0	2/2
D11S654	11p12–11p11.2	Unknown	2/0	1/1
D3S1560	3p26–3p25	$(AC)_{22}$	2/1	2/1
D3S1286	3pter–3p24.2	$(T)_5$	7/4	3/3
D3S1289	3p23–3p21	$(CA)_{23}$	5/1	5/4
D17S695	17	$(AAAG)_n$	3/3	1/1
D17S654	17	$(CA)_n$	5/3	1/1
D17S656	17	Genbank accession no. 2574	1/0	1/0

[a] Locations listed as chromosome number only have no more specific information available in current web genomic data bases.
[b] T, tumor; S, saliva.

Sciences, Inc., Arlington Heights, IL) and T4 kinase (New England Biolabs, Inc., Beverly, MA) in a total volume of 50 µl. PCR reactions were carried out in a total volume of 12.5 µl containing 10 ng genomic DNA, 0.2 ng labeled primer, and 15 ng of each unlabeled primer. The PCR buffer included 16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM magnesium chloride, 10 mM β-mercaptoethanol, and 1% DMSO, to which were added 1.5 mM deoxynucleotide triphosphates and 1.0 unit Taq DNA polymerase. (Boehringer-Mannheim Biochemicals, Indianapolis, IN). PCR amplifications of each primer set were performed for 30–35 cycles consisting of denaturation at 95°C for 30 s, annealing at 50–60°C for 60 s, and extension at 72°C for 60 s. One-third of the PCR products were separated on 8% urea-formamide-polyacrylamide gels and exposed to film from 4 to 48 h. For informative cases, allelic loss was documented if one allele was significantly decreased (>50%) in tumor- or oral-test DNA compared with the same allele in the normal (lymphocyte) DNA. MSI was described if an additional band representing a change in repeat number was noted in tumor- or oral-test DNA. All of the samples were assessed by two observers independently (M. F. S., D. S.) and borderline cases were decided by densitometry. All of the identified alterations were confirmed by repeating the PCR reaction and electrophoresis. Samples were coded, so that the observers were blinded to the individual subject.

RESULTS

Thirty-eight of 44 tumors (86%) displayed a microsatellite alteration in at least one locus (Table 3). Twenty-five displayed MSI and 31 had LOH at an examined locus. Eighteen tumors displayed both MSI and LOH, 13 had LOH only and 7 had MSI only. Thirty-one tumors had microsatellite alterations at more than one locus, but 7 tumors displayed changes in only one marker. All of the 23 microsatellites were altered in at least one tumor. With this panel, the average number of alterations per tumor was 2.7 (range, 0–13) with an average of 1.5 instances of LOH (range, 0–5) and 1.2 of MSI (range, 0–13). Thirteen markers displayed MSI in two or more tumors (maximum, six tumors). Five microsatellites were the only markers altered in at least one tumor (Table 2).

Table 3 Number of tumors with LOH and MSI

Two by two tables demonstrate the number of tumors and oral samples with genetic alterations at various microsatellite loci. Tumors (6) lacking both MSI and LOH had no markers available for screening the oral exfoliative samples.

	Tumors with MSI No	Yes	
Tumors with LOH			
Negative	6	7	13
Positive	13	18	31
	19	25	44

None of the oral specimens of the tumor-free controls displayed a microsatellite alteration. In contrast, in 35 (92%) of 38 cancer cases for which a tumor marker was available, at least one genetic alteration matching those seen in the tumor was also

Table 4 Number of tumors and oral samples with any microsatellite alteration

Of 38 cases in which the tumor had at least one microsatellite marker, cells with the same alteration were identified in the test sample in 35.

	Tumors with alteration		
	No	Yes	
Oral sample			
Negative	6	3	9
Positive	0	35	35
	6	38	44

Table 5 Number of tumors and oral samples with LOH

At least one marker with LOH was present in the tumor in 31 cases and could be identified in a test sample in 19 cases.

	Tumors with LOH		
	No	Yes	
Oral sample			
Negative	13	12	25
Positive	0	19	19
	13	31	44

seen in an exfoliative oral-test specimen (35/44, 79% overall; Table 4). In cases for which the oral rinse and swab specimens were analyzed separately, tumor DNA was detected in 57% of the swab samples compared with 44% of the rinse samples. This difference in rate of detection was not statistically significant. All of the three cases displaying tumor markers that were not detected in the oral exfoliative sample harbored tumor in the oropharynx, one involving the tonsil, one, the base of tongue, and one, the posterior pharyngeal wall (staged T_3, T_1, and T_2, respectively). In two of these cases, only one marker with LOH was found in the tumor, and, therefore, only one was potentially available for detection.

MSI is more easily recognized amid a background of normal cell DNA than is LOH. As a result, MSI was more efficient for tumor cell detection in the oral samples. Of the 31 cases in which a tumor displayed LOH, the same loss was detected in the matching test sample in 19 cases (61%; Table 5). In contrast, MSI was detected in the test specimen in 24 (96%) of 25 cases in which a shifted marker was present in the tumor (Table 6). Overall, MSI contributed 64% of the positive tumor-test matches in the sample set.

Twelve (92%) of 13 small primary (T_x and T_1) lesions for which a microsatellite marker was identified in the tumor were also detected in the test sample (12 of 16 overall; Table 1). These 12 included the three cases that presented with metastatic HNSCC in cervical lymph nodes without a detectable primary lesion. In all three of the cases, cells matching the metastatic tumor were detected in the test sample. Nine of 10 T_1 tumors were also detected, including one located in the supraglottic larynx. The relatively small size of T_x and T_1 primary lesions did not adversely influence the likelihood of detecting a LOH marker in saliva. Of the 13 T_x and T_1 lesions displaying at least

Table 6 Number of tumors and oral samples with MSI

At least one shift marker was present in the tumor in 25 cases and could be identified in the test sample in 24 cases.

	Tumors with MSI		
	No	Yes	
Oral sample			
Negative	19	1	20
Positive	0	24	24
	19	25	44

one LOH marker, the same alteration was detected in saliva of 6 (60%).

Index microsatellite alterations were identified in the tumor of 4 of 6 patients entered in the study after prior radiation therapy. Matching alterations were detected in exfoliative oral samples in all four of the cases.

DISCUSSION

Early cancer detection in body fluids such as urine or sputum typically requires morphological identification of a few neoplastic cells in a background of normal epithelial cells. Standard cytological methods have been shown to have potential value for the detection of second primary tumors in the esophagus of patients with HNSCC (9), and have been assessed for the early detection of lung cancer (10). We did not subject our samples to cytological evaluation, although the dental literature of the 1960s and 1970s contained numerous reports on the use of oral cytology as a diagnostic approach. However, low sensitivity and specificity precluded the general adoption of microscopic cytology for the detection of primary or recurrent HNSCC (11, 12, 13). Cytological evaluation is labor intensive, requiring a high degree of experience to accurately identify morphologically suspicious cells. In contrast, assessment of molecular alterations is less subjective, relying on the identification of tumor-specific changes in the DNA. This approach is also amenable to automation using recent innovations in microcapillary array technology (14). In our hands, molecular markers displaying instability have proven to be highly sensitive, able to detect a single tumor cell out of 200 normal cells in the urine; however, LOH markers are much less sensitive, able to detect only one neoplastic cell among 3–4 normal cells (5). In patients with bladder cancer, large amounts of neoplastic cells are shed into the urine. We were concerned that the rate of normal cell turnover in the oral cavity would produce more exfoliation of normal cells obscuring the detection of cancer beyond that seen in the bladder. However, the results of this study have shown that microsatellite analysis (both LOH and MSI) can be used to detect DNA from exfoliated tumor cells in the saliva of cancer patients.

The panel of 23 microsatellites identified at least 1 altered marker for all but 6 of the tumors in this series. Only tumors that displayed alterations at one or more of the loci could be detected in an exfoliative sample, which excluded 14% of tumors from the analysis. When a tumor marker was available, exfoliated cancer cells were detectable in the oral specimens from the vast majority (92%) of cases. Failure to detect tumor cells in three

cases displaying a tumor-specific marker may be attributed to a number of factors, such as necrotic tumor yielding little DNA, a high background of normal epithelial cells, mostly submucosal tumor growth, problems with sample handling and processing, or the type (LOH *versus* MSI), and quantity of altered markers.

Previous studies have demonstrated the capability of scrapings from oral lesions (15) and cytological brushings of laryngeal neoplasms (16) to produce cells with tumor-specific microsatellite alterations. Our protocol included swabbing the tumor bed, but we did not vigorously scrape or brush the index lesion when it was visible. Instead, the harvesting of exfoliative cells was performed in a manner intended to simulate a potential screening protocol. Our population included six cases in which the tumor was not in a region that could be sampled in an office-based swab protocol. The hypopharynx and larynx are easily sampled under general anesthesia, and exfoliative samples from these areas might be obtained in a manner similar to "induced sputum" cytology (17). If these cases were eliminated from the analysis, the protocol was able to detect cancer in 29 (90%) of 32 oral and oropharyngeal cases for which a tumor marker was available, or 76% of the remaining population of 38 patients overall.

This work constitutes a feasibility trial aiming to demonstrate proof of principle for a molecular detection approach using our panel of microsatellite markers in patients with obvious HNSCC. If the test is validated and refined, molecular analysis of exfoliated oral mucosal cells may be useful as a detection method for patients at risk for developing HNSCC *de novo*, and as a surveillance tool for patients after completion of therapy. A swab of the tumor bed could be performed after treatment for tumor surveillance, but swabbing the tumor will not be possible when the saliva test is applied to screen at-risk individuals without clinical lesions. An indication of the applicability of the saliva test for screening the at-risk population is found in the successful test results of the three patients with metastatic HNSCC from unknown mucosal primary sites. Oral rinses from these patients produced exfoliated cancer-related cells in each case. Although the oral sample could not identify the site from which the clonal cells originated, it could identify their presence. Directed biopsies of commonly involved mucosal sites are typically harvested in the work-up of patients with HNSCC of unknown primary. These could then be analyzed both histologically and using microsatellite analysis. We have reported the utility of molecular testing to identify cancer cells in histologically benign directed biopsy samples from such patients (18).

Radiation therapy might prohibit the application of the test in the surveillance setting by altering the rate of exfoliation of both normal and cancer cells in the upper aerodigestive tract or by interfering with the identification of microsatellite alterations. However, successful detection of tumor cells was possible in the test samples of all four of the patients who had undergone previous radiation therapy and who had a marker identified in their tumor. These results support the potential application of the test in postradiation therapy surveillance.

Our panel of markers has been developed with an emphasis on MSI detection and known areas of LOH, featuring a majority of tetranucleotide repeat markers with the $(AAAG)_n$ repeat motif (Table 2). It was not our goal to streamline the panel of markers at this stage of test development. Given the limited sample size of this pilot cohort, a marker altered in even one tumor was considered potentially valuable. Five microsatellites were the sole markers available for at least one tumor and only two microsatellite alterations had no positive matches between tumor and test samples. Thus, the panel contained few, if any, unnecessary markers. For example, one marker (D20S85), that was detected in only one tumor was also the only available marker for that tumor and was detected in the test sample as well. Markers that display MSI were found to be better than LOH markers for detecting tumor cells in a background of normal cells. Relatively pure clonal populations of tumor cells are required for detection of LOH, because the loss of the signal may be obscured easily by the presence of the alleles in the normal cell majority. Microsatellite loci that display MSI are valuable because the amplified novel tumor DNA band separates from maternal and paternal alleles on gel electrophoresis and serves as a unique positive-signal clonal marker for the tumor and its exfoliated daughter cells. Thirteen markers displayed MSI in multiple tumors. These microsatellites are arguably the most valuable in the panel. If altered in tumor, they were detected in saliva 66% of the time and account for 47% of all of the positive saliva results in the entire study.

Future refinement of the approach may focus on several areas. More microsatellite markers could be added to the panel, thus increasing the yield of detectable tumors above the current 86%. In particular, tetranucleotide markers displaying MSI in a high proportion of tumors seem to be promising candidates for addition to the panel. Tumor-specific promoter methylation markers may also be useful (19). Microcapillary array technology now allows more rapid and efficient screening of large numbers of samples (12).

The molecular assessment of exfoliated cells from oral rinsing/swabbing must now be tested in a prospective, blinded fashion in clinical settings requiring actual cancer detection. The fact that no healthy control subject had any DNA alteration is encouraging, indicating the excellent specificity of the microsatellite analysis. Normal appearing mucosa in patients with early cancer or premalignant lesions has been shown to harbor occult microsatellite alterations (20). It is precisely in these patients that the value of detecting asymptomatic cancers by microsatellite analysis must be prospectively assessed, a setting requiring a highly specific test. A saliva test could be administered by nonspecialists in remote locations as a screening tool to select patients for referral for careful evaluation of the upper aerodigestive tract. Finding early stage, previously undetected disease and prompt identification of persistent disease after therapy using sensitive microsatellite analysis may ultimately save lives.

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APPENDIX D

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›etection of *APC* and *k-ras* Mutations in the ›erum of Patients with Colorectal Cancer

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ABSTRACT: Detection of tumor DNA in peripheral blood of patients with colorectal cancer (CRC) may allow early diagnosis of tumor disease and be of prognostic value. However, a reliable tumor marker detectable in the serum of patients with this disease is missing. Because *k-ras* and *APC* mutations occur frequently and at an early stage in CRCs, these mutations might also be detected in the serum of CRC patients and serve as tumor markers. Hence, tumor tissues of CRC patients were examined for the presence of mutations in the *k-ras* and *APC* genes. If a mutation was detected in the tumor, the serum of the patient was screened subsequently for the presence of this mutation. *K-ras* mutations were detected in 22 of 30 colorectal tumor tissues, but only in six patients was the mutation identified in their serum samples. Mutations of the *APC* gene were identified in 25 of 65 tumors; 20 of these 25 patients showed the respective mutation in their serum. Given their higher detection rate, *APC* mutations could be a more informative serum marker than *k-ras* in CRC patients.

KEY WORDS: *APC* mutations, colorectal cancer, *k-ras* mutations, serum marker.

e presence of tumor DNA in the plasma of cancer ;ients was observed more than two decades ago.[1] e presence of extractable amounts of DNA in the ısma of patients with a variety of malignancies and absence in healthy controls could be demonstrated.[2] rthermore, higher amounts of serum DNA in pants with metastatic tumor disease than in patients th localized disease and decreasing amounts of DNA er tumor regression have been shown.[1] However, is only since publication of the milestone papers on ; detection of tumor DNA in the plasma of patients th small-cell lung cancers and head and neck canrs[3,4] that an ever-increasing number of articles on ; detection of tumor DNA in the serum or plasma of ncer patients have been published.[5–8] This approach ay lead to improvement in the diagnosis of cancers several ways: (1) The detection of circulating tumor NA may be a marker of metastasis and lead to a ore precise staging of tumor disease, thus serving a prognostic marker; (2) searching for tumor DNA serum may be useful for detecting recurrent disease ter surgery or for planning chemotherapy; and (3) rculating tumor DNA may prove to be a useful tumor arker in cancer screening.

In studies on the detection of tumor DNA in the serum of colorectal cancer (CRC) patients published thus far, microsatellite instability (MSI), loss of heterozygosity, *k-ras* mutations, and *p53* mutations were chosen as diagnostic parameters. MSI has been shown to be a valuable serum marker in patients with small-cell lung cancer and head and neck cancers.[3,4] However, MSI has been shown to be present in only 10% to 15% of CRC patients.[9] Furthermore, in none of 15 tumors showing MSI and in none of 31 tumors with loss of heterozygosity could Hibi et al.[8] trace these microsatellite variations to the sera of their patients.

K-ras mutations are common in CRC, and approximately 80% of the mutations are found in codon 12 of the gene.[10,11] Data regarding *k-ras* mutations in the serum of CRC patients is contradictory. Though some investigators were able to detect mutant *k-ras* in the serum of 86% of CRC patients whose tumors harbored a *k-ras* mutation,[5,6] Hibi et al.[8] found a mutation in the serum in only 3 of 16 cases. In contrast, the latter authors were able to detect *p53* mutations in the serum of 7 of 10 patients whose tumors had a *p53* mutation. However, a *p53* mutation was detected in only 10 of a total of 33 CRC patients. The authors conclude that

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the use of *p53* is limited by the large number of different mutations within the gene and that other genetic targets such as *APC* might be a useful marker to study.

It is well-known that somatic *APC* mutations occur as first events in the cascade of molecular alterations within the adenoma-carcinoma sequence.[12] Germline mutations in the *APC* gene are responsible for familial adenomatous polyposis, an autosomal dominant precancerous condition characterized by the appearance of hundreds to thousands of colorectal adenomas. *APC* is a relatively large gene (>8 kb), and germline mutations in familial adenomatous polyposis patients are widely spread over major parts of its coding sequence. Most somatic mutations detected in sporadic CRC, however, are limited to the so-called mutation cluster region (MCR) in exon 15, encompassing codons 1286 through 1513.[13] Mutations occurring in this relatively small part of the gene can easily be detected by heteroduplex or single-strand conformation analysis.

In this study, we set out to compare the potential of *k-ras* mutations and *APC* mutations as serum markers in patients with CRC.

MATERIALS AND METHODS

Patients

We included in the study 65 patients with CRC after obtaining their written informed consent. All tumors were histologically characterized as adenocarcinomas and were classified according to Union Internationale Contre le Cancer (UICC) stages: 23 patients had UICC stage I tumors, 7 stage II, 31 stage III, and 4 stage IV tumors, respectively. Peripheral blood samples were obtained 1 day before and at two intervals (1 and 10 days) after surgical resection of the tumor. Serum was prepared immediately and stored at −70°C.

DNA Extraction

Tumor DNA of the CRC patients was extracted from paraffin-embedded microdissected tumor tissue using the QiaAmp Tissue Kit (Qiagen, Hilden, Germany). DNA from serum samples was extracted by the QiaAmp Blood & Body Fluid Protocol (Qiagen).

Detection of *k-ras* Codon 12 Mutations

Tumor DNA was amplified by polymerase chain reaction (PCR) using the primers and PCR conditions as described by Jiang et al.[14] and Enrius et al.[15] The forward primer (primer A: 5′-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT-3′) contains a mismatched nucleotide at the 3′ end that generates a restriction site for BstNI in wild-type DNA but not in the DNA that harbors a mutation at codon 12. DNA of the colorectal tumor cell line SW480 was used as a positive control for the *k-ras* codon 12 mutation. PCR products were digested with BstNI and separated on polyacrylamide gels followed by silver staining.

Detection of *APC* Mutations

Somatic mutations in the MCR of the *APC* gene (codons 1286–1513) in the tumors were detected by heteroduplex and single-strand conformation analysis followed by silver staining, as described previously (Figure 1).[16] If a mutation was detected, the existence of this mutation was excluded in the control DNA

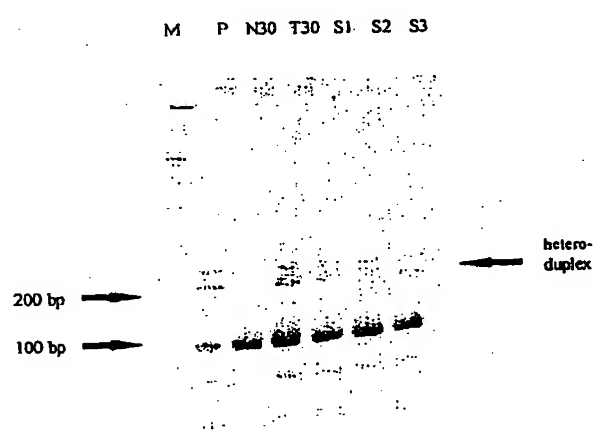


FIGURE 1. Detection of the 5 bp deletion at codon 1309 of the *APC* gene in tumor tissue (T30) and in serum samples of patient 30 taken 1 day before (S1), 1 day after (S2), and 10 days after operation (S3). The heteroduplex bands are not present in DNA from lymphocytes of the patient (N30). (P = patient with familial adenomatous polyposis, with a germline mutation at codon 1309, as a positive control for the mutation; M = molecular weight marker with 100 bp ladder.)

d from the leukocytes of the same patient to rule germline *APC* mutation.

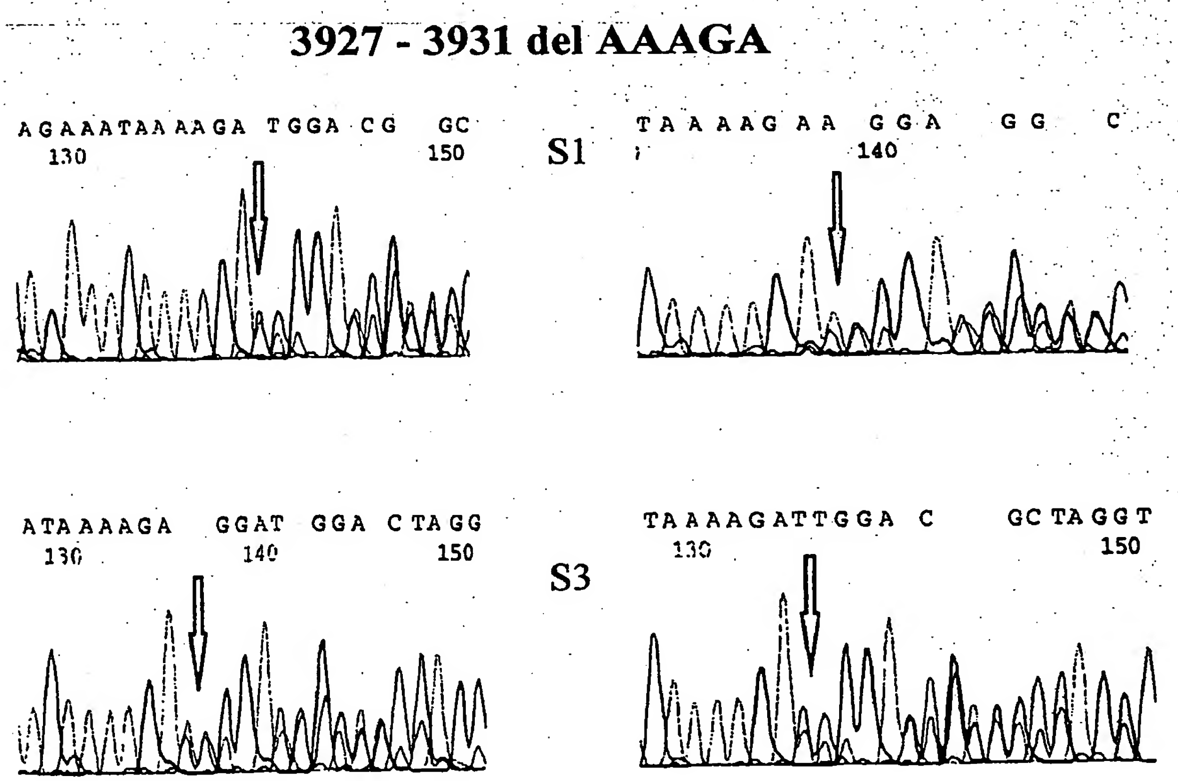
ction of Tumor DNA in m Samples

ı the patients whose tumors exhibited *k-ras* or nutations, we screened their serum samples for esence of these mutations. Essentially, the same ion methods were applied in the sera as in the 's for *k-ras* or *APC* mutations. However, owing well-known fact that the tumor DNA present serum of cancer patients is partially degraded,[17] [CR of the *APC* gene was examined in seven ıpping fragments with a length of approximately ıp each. (Primers and PCR conditions can be ıed on request.) Mutations detected by this ıd were subsequently confirmed by sequencing serum DNA on an ABI 377 sequencer (Perkin ; Weiterstadt, Germany) (Figure 2).

In cases in which the mutations could not be traced to the sera, a reamplification was performed as described by Deuter and Müller.[18] Briefly, the portion of the polyacrylamide gel corresponding to the presence of aberrant bands in the respective tumors was excised and incubated with water at 60°C for 1.5 hours; 5 μl was reamplified, using the same primers and PCR conditions as outlined for *APC* mutations and primer set A and D (primer D: 5′-TCA TGA AAA TGG TCA GAG AAA CC-3′) for *k-ras* mutations.

RESULTS

We examined CRC tissue from 65 patients for *APC* and *k-ras* mutations that might be detectable in the patients' sera. The presence of tumor-specific mutations was followed up in the three serum samples obtained from each patient, 1 day before and 1 and 10 days after tumor excision.



IRE 2. Sequencing of the DNA derived from tumor tissue (T) and serum samples of patient 30 ı 1 day before (S1), 1 day after (S2), and 10 days after operation (S3).

Detection of *k-ras* Mutations

In a first step, 30 CRC patients were examined. By using the mutant allele–specific restriction method,[14,15] we were able to detect mutations in codon 12 of the *k-ras* gene in 22 of the 30 tumors (73%; Table 1). However, when the same method was applied to the serum of the patients, the *k-ras* mutation was detectable in only one patient. To increase the detection rate in the serum samples, the position characteristic of the mutant allele was excised from the gel, eluted, and reamplified.[18] After reamplification, the mutation was detectable in the serum samples of another five patients. Thus, we ended with a detection rate of 27% (6 of 22 patients). In all six patients in whom the *k-ras* mutation could be traced to the serum, all three serum samples showed the mutation (see Table 1).

Detection of Mutations in the *APC* Gene

We also screened the tumors for mutations in the MCR of the APC gene. In 13 of the 30 patients (43%), a mutation was detected in the tumor. In 11 of these 13 patients, the mutations could be traced to the serum (see Figure 1). In one serum sample, no PCR product could be obtained. In six cases, the aberrant bands observed by heteroduplex analysis of the serum samples were excised from the gel and sequenced, to prove that they are due to the same mutations as those detected in the tumors (see Figure 2).

From this series of experiments, we determined that *k-ras* mutations were present in the majority of CRC cases (22 of 30; 73%) but could be detected in only a minority of the serum samples (6 of 22; 27%). On the other hand, somatic *APC* mutations were de-

TABLE 1
Detection of *k-ras* Mutations in Colorectal Tumors and Serum Samples of 22 of 30 Patients

Patient	UICC stage	Tumor	Serum S1	Serum S2	Serum S3
T7	I	+	–	–	–
T20	I	+	+(GE)	+(GE)	+(GE)
T23	I	+	–	–	–
T26	I	+	–	–	–
T28	I	+	–	–	–
T4	II	+	+	+	+
T6	II	+	–	–	–
T9	II	+	–	–	–
T11	II	+	+(GE)	+(GE)	+(GE)
T27	II	+	–	–	–
T8	III	+	–	–	–
T15	III	+	+(GE)	+(GE)	+(GE)
T16	III	+	NPP	NPP	NPP
T21	III	+	–	–	–
T22	III	+	–	–	–
T24	III	+	+(GE)	+(GE)	+(GE)
T29	III	+	+(GE)	+(GE)	+(GE)
T30	III	+	–	–	–
T31	III	+	–	–	–
T32	III	+	NPP	NPP	NPP
T3	IV	+	–	–	–
T18	IV	+	–	–	–
		Σ22	Σ6	Σ6	Σ6

UICC = Union Internationale Contre le Cancer; S1 = serum 1 day before operation; S2 = serum 1 day after operation; S3 = serum 10 days after operation; + indicates mutation detected; – indicates no mutation detected; (GE) = detectable after extraction from gel and reamplification; NPP = no polymerase chain reaction product.
Note: No *k-ras* codon 12 mutation was identified in eight tumors.

ı only 43% of all tumors (13 of 30) but could d to most of the corresponding serum samples 13; 85%). Thus, *APC* mutations were more ıly detectable in the serum of our CRC patients, ıng that mutant *APC* might be a more sensi- nor marker as compared to *k-ras* codon 12 ns.

ɜrefore, in a second series of 35 CRC patients, nined tumor and serum samples for APC muta- ly. In 12 tumors, an *APC* mutation was detected ıld be traced to the corresponding serum sam- 9 cases. Thus, the results were similar to those d in the first 30 patients (Table 2).

our study, we observed a tendency toward a stage-dependent difference in the occurrence of *APC* mutations in the serum of CRC patients, which may point to its possible significance as a prognostic marker. The percentage of *APC* mutations detected in the tumors did not differ among UICC stage I, II, III, and IV tumors: Hence, the well-known early occurrence of mutations in the *APC* gene has been confirmed. Overall, *APC* mutations have been detected in 25 of the 65 tumors examined (see Table 2). Subsequently, the serum samples of these 25 patients were screened for the presence of the respective mutations. In three patients whose *APC* mutations could not be detected in their serum samples, no PCR products were obtained at any of the three blood sampling events,

E 2
tion of *APC* Mutations in Colorectal Tumors and Serum Samples of 25 Patients

ıt	UICC stage	Tumor	Serum S1	S2	S3	Sequencing of serum DNA
	I	+	+	+	+	ND
	I	+	+	+	–	ND
	I	+	+	+	+	nt4372 del C
	I	+	+	+	+	nt4087-4088 del AA
	I	+	NPP	NPP	NPP	
	I	+	–	–	–	
	I	+	+	+	–	ND
	II	+	+	+	–	ND
	II	+	–	–	–	ND
	II	+	+	+	+	nt4087-4088 del AA
	II	+	+	+	+	ND
	III	+	+	+	+	nt4463 del T
	III	+	NPP	NPP	NPP	
	III	+	+	+	+	ND
	III	+	+	+	+	nt3927-3931 del AAAGA
	III	+	+	+	+	ND
	III	+	+	+	+	ND
	III	+	NPP	NPP	NPP	
	III	+	+	+	+	ND
	III	+	+	+	+	nt3927-3931 del AAAGA
	III	+	+	+	+	ND
	III	+	+	+	+	ND
	IV	+	+	+	+	nt3927-3931 del AAAGA
	IV	+	+	+	+	ND
	IV	+	+	+	+	ND
		Σ25	Σ20	Σ20	Σ16	Σ7

= Union Internationale Contre le Cancer; S1 = serum 1 day before operation, S2 = serum 1 fter operation, S3 = serum 10 days after operation; + indicates mutation detected; – indicates ɹtation detected; ND = not done; NPP = no polymerase chain reaction product.
In 40 tumors, no mutation in the mutation cluster region of the *APC* gene could be detected. Of tumors, 10 were UICC stage I, 8 UICC stage II, 14 UICC stage III, and 8 UICC stage IV.

possibly owing to the presence of inhibitors in the patients' serum. Of the remaining 22 patients whose tumors showed *APC* mutations, 10 had UICC stage I and II tumors and 12 had UICC stage III and IV tumors. Whereas in all 12 patients with stage III and IV tumors the respective *APC* mutation could be traced to their serum samples, the mutations could be detected in only 8 of the 10 patients with stage I and II tumors. Furthermore, in four of these eight patients, the respective mutation was no longer detectable in the serum sample taken at day 10 postoperatively. In contrast, none of the 12 patients at stages III and IV had lost the *APC* mutations from their serum taken at day 10 postoperatively (see Table 2). However, any comment on the significance of this difference must be prefaced by information on follow-up of these patients.

DISCUSSION

According to the literature, 20% to 50% of all CRCs harbor somatic mutations in the *k-ras* gene, and 80% of these occur in codon 12 of the gene. By the mutant allele–specific restriction method we used, we were able to detect mutations in codon 12 of the *k-ras* gene in 22 of 30 tumors (73%; see Table 1). However, the mutation could be traced to the serum of only 6 of these 22 patients (27%). This finding is in agreement with the data published by Hibi et al.,[8] who found *k-ras* mutations in the serum in 3 of 16 CRC patients (19%) whose tumors exhibited *k-ras* mutations. In contrast, studies by Anker et al.,[5] de Kok et al.,[6] and Kopreski et al.[7] found a much higher proportion of mutant *k-ras* in the serum of CRC patients. Kopreski's group[7] reported on the detection of *k-ras* codon 12 mutations in the serum of 12 of 31 patients (39%), all of whom had metastatic disease.

Likewise, de Kok et al.[6] included in their study only patients with advanced disease (UICC stage IV tumors). They found *k-ras* codon 12 or 13 mutations in the serum of six of seven CRC patients (86%) whose tumors proved positive for a mutation. Anker et al.[5] also found mutations in the serum of six of seven patients whose tumors harbored *k-ras* mutations. In a subset of their patients, Anker's group[5] proved the presence of *k-ras* mutations in the serum through cloning and sequencing techniques to rule out the possibility of false-positive results.

False-positive results in the detection of *k-ras* mutations are due to the generation of PCR artifacts, which are likely to occur with increasing cycles of PCR. Therefore, the method of reamplification of the mutant allele we used could be prone to false-positive results, as a second round of 25 PCR cycles is used after the first PCR round consisting of 32 cycles. To rule out this possibility, the existence of the detected mutations should be confirmed by a second method (e.g., sequencing) in an independent PCR probe. However, this would make the diagnostic program even more laborious and expensive and not feasible in a routine setting. Furthermore, we could find *k-ras* mutations in the serum of only 27% of the patients, which confirms the results of Hibi et al.[8] Thus, we decided to complete the *k-ras* investigations after 30 patients and proceed with the *APC* study, as the results of the latter were more promising.

By screening colorectal tumors of 65 patients for somatic mutations in the MCR of the *APC* gene, we detected a mutation in 25 tumors (38.5%; see Table 2). This rate of mutations within the MCR agrees well with published data.[13,18,19] In 20 of the 25 cases, the respective mutations could be detected in the serum. In our hands, the screening for mutations within the MCR of the *APC* gene by nonradioactive single-strand conformation polymorphism and heteroduplex analysis is both rapid and simple, and the results obtained with mutated *APC* as a serum marker in CRC patients were more promising than results obtained with *k-ras*. Furthermore, we observed a tendency to a stage-dependent difference in the occurrence of *APC* mutations in the serum of CRC patients, which may point to a possible significance as a prognostic marker.

There were two considerations for taking the serum samples at three different time points: First, it is possible to detect tumor DNA in the serum postoperatively but not preoperatively, owing to the dissemination of tumor cells during surgical intervention. However, in all patients in whom we were able to detect mutant DNA in the serum, the mutant DNA was present both 1 day before and 1 day after tumor excision. We therefore conclude that this method is not useful for the demonstration of tumor cell dissemination by the surgeon.

Second, it is possible that mutations detected in the serum preoperatively are lost after surgery as an indication of complete tumor excision. Although we observed this, it happened in only a minority of cases and, even in four of ten patients at UICC stage I

tumors, the mutant DNA remained 10 days eratively. We therefore speculate that the last samples might have been taken too early, as me may be needed for the mutant DNA to clear ie blood. However, the time point was chosen ctical reasons, because the patients usually stay clinic for approximately 10 days after surgery. ken together, despite the much higher propor- *k-ras* mutations detected in tumors (73%) as red to *APC* mutations (38.5%), in our hands ; superior as a serum marker in patients with as we could trace the *k-ras* mutations to the of only 27% of the patients, whereas we could ie *APC* mutations detected in the tumors to 80% corresponding serum samples. In conclusion, eems to be a good serum marker for the detection or cell DNA from CRCs. Nonetheless, there s a need to identify additional markers, as only r so of all CRCs harbor mutations within the of the *APC* gene. To estimate whether this serum is also a valuable indicator of prognosis in patients, a longitudinal follow-up study of the s is being conducted.

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APPENDIX E

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olated Tumor Cells Are Frequently Detectable in ie Peritoneal Cavity of Gastric and Colorectal ancer Patients and Serve as a New rognostic Marker

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jective

evaluate the prognostic significance of isolated tumor cells ected by a panel of various monoclonal antibodies.

nmary Background Data

viously, we showed by using immunocytology that cancer s are frequently found in bone marrow and peritoneal cav- amples of gastrointestinal cancer patients.

thods

lings in bone marrow and peritoneal cavity samples were npared and correlated with the 4-year survival rate of 84 tric and 109 colorectal patients with cancer.

Results

Although positive results in the bone marrow showed little prognostic significance, the peritoneal cavity results correlated with the 4-year survival rate (gastric cancer: $p = 0.0038$; colorectal cancer: $p = 0.0079$). Additionally, in subgroups of patients with early (gastric cancer: $p = 0.02$, colorectal cancer: $p = 0.48$) and advanced (gastric cancer: $p = 0.02$, colorectal cancer: $p < 0.0001$) tumor stages, a correlation of immunocytologic findings and the survival rate was seen.

Conclusions

The detection of minimal residual disease in the peritoneal cavity serves as a new prognostic marker.

The success of surgical treatment in patients with gastric colorectal cancer is often limited. This is because of al recurrence or the development of distant metastases or itoneal carcinosis by cells that have already been seeded he time of operation but cannot be detected using con- tional diagnostic tools. The elimination of these micro- tastatic cells is the aim of various adjuvant therapies,[1,2] obviously it would be helpful to detect minimal residual ease.

Jsing immunocytologic methods, which are significantly re sensitive than conventional cytology,[3] it has become sible to detect disseminated tumor cells in the bone rrow of patients with breast cancer,[4] small cell lung cancer,[5] neuroblastoma,[6] prostatic cancer,[7] gastric cancer,[8] colorectal cancer,[9] and pancreatic cancer.[10]

Bone marrow metastases are rare in gastric and colorectal cancer.[11] The high frequency of intraperitoneal tumor relapse and peritoneal carcinosis strongly suggests that micrometastatic cells are most likely present within the peritoneal cavity.

Previously, we showed that disseminated cancer cells become specifically detectable in the peritoneal cavity of patients with gastric, colorectal, and pancreatic cancer.[10] It was shown that at the time of the operation, tumor cells occur with high frequency in the peritoneal cavity and in the bone marrow. However, it remains unclear if single tumor cells are of prognostic significance and have the ability to form metastatic disease.

No comprehensive immunocytologic studies exist concerning the prognostic significance of isolated intraperitoneal tumor cells. Therefore, we extended our former study

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ırger collection of patients and investigated peritoneal ɛ samples collected at the beginning of the operation. dian, the follow-up was 4 years. We also examined the marrow for micrometastatic cells because this is an ' accessible filter compartment of the bloodstream, and ›mpared the results of these two compartments of the

munocytologic studies of bone marrow samples from ːr patients used anticytokeratin antibodies, which stain ːlial cells not present in bone marrow.[4,5,9] This is not icable for peritoneal cavity samples because of cross- on with mesothelial cells. We applied and compared a of different monoclonal antibodies (anti-CEA, anti-)-9, anti-Ra96, anti-C54-0, anti-17-1A) directed against r-associated antigens that have been tested in a control) and do not react with normal peritoneal and bone ›w cells. Bone marrow samples were also studied with nticytokeratin antibody Kl-1.

ː showed that tumor cells were frequently detectable in eritoneal cavity and in the bone marrow. Their occur- ː in the peritoneal cavity correlated to a highly signif- degree with the postoperative survival rate of colorec- ıd gastric cancer patients.

ΓERIAL AND METHODS

ents

l patients were extensively informed and gave written ːnt for the investigations, including the bone marrow ation. The study was approved by the ethical commis- of the University Hospital Kiel.

ʒhty-four gastric and 109 colorectal patients with can- vho underwent surgery were investigated. No bone ›w sample was obtainable in 26 patients because they ned to give consent. Peritoneal lavage could not be ›rmed in 22 patients because of adhesions.

control group comprised 58 patients with a variety of ıalignant diseases, including benign liver tumors (n = ːigmoid diverticulitis (n = 8), chronic pancreatitis (n = holecystolithiasis (n = 4), duodenal ulcers (n = 4), .asia (n = 4), and hypersplenism (n = 4). Forty-five marrow and 43 peritoneal cavity samples were col- d from the control group. Also, ascites from patients liver cirrhosis (n = 5) and bone marrow samples from nts with benign hematologic diseases (n = 12) were ːtigated.

ıples

›ne marrow (8 mL) was aspirated from the right spina ı anterior at the beginning of the operation using a hidi needle. Peritoneal lavage was performed before pulation of the tumor. One liter of isotonic sodium ide solution was instilled and immediately removed. lavage solution was centrifuged (1200 g for 10 minutes). The cells were further processed by Ficoll-Paque (Pharmacia, Uppsala, Sweden) and were centrifuged onto microscopic slides (2.5 $\times$ 10^5 cells/slide). Cytospins were fixed in acetone and stored at −20 C.

Immunocytochemistry

Staining of cytospins was performed by the immunoperoxidase method with six different monoclonal antibodies, as described previously10: 1) C1P83[12]: anti-CEA; 2) CA19-9[13]: determinants of Lewis blood group antigens; 3) 17-1A[14]: membrane antigen; 4) Ra96[15]; mucin; 5) C54-0[16]: membrane antigen (because of a cross-reactivity with a subpopulation of lymphopoietic cells in the bone marrow, C-54-0 was used only in peritoneal cavity samples); and 6) Kl-1 (Dianova, Hamburg, Germany): cytokeratin (used only in bone marrow samples). Each antibody was tested on a slide with 2.5 $\times$ 10^5 cells. A positive control (WIDR–colon cancer cells) and a negative control (no specific monoclonal antibody) were stained in parallel to the samples. The microscopic evaluation was carried out independently by two investigators who were unaware of the patient data.

Evaluation of Data

Samples were evaluated as positive for tumor cells if at least one cell reacted with one of the monoclonal antibodies. The detection rate was correlated with the UICC classification of the tumor stage and the R classification.[17]

After surgery, patients were examined either in our outpatient clinic or by their general practitioner. Every 3 months a clinical examination and blood tests, including tumor markers CEA and CA19-9, were done, and every 6 months a sonography or CT scan and an endoscopy were performed.

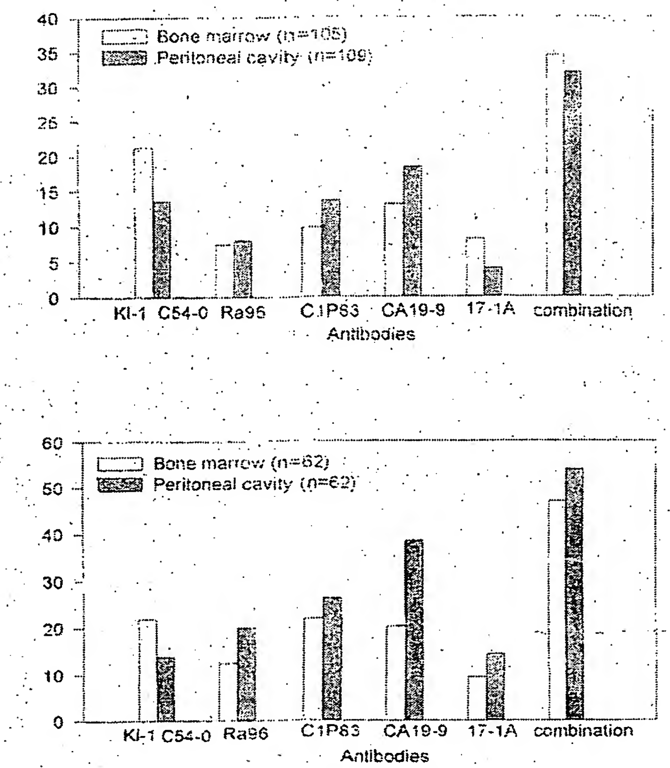
Survival rates were determined by Kaplan-Meier test and calculations of significance by the log-rank test.

RESULTS

Control Group

In 43 of 45 patients, no cell staining was seen in bone marrow samples with Kl-1, C1P83, Ra96, CA19-9, and 17-1A. In 2 patients, single cells were stained with Kl-1, C1P83, and 17-1A. Also, 1 patient had positive cells for CA19-9. Both patients were strongly suspected of suffering from pancreatic cancer and were therefore treated by a Whipple operation. The histologic analysis could not confirm this diagnosis and found chronic pancreatitis.

The peritoneal lavage samples of patients with no malignant diseases showed, in 40 of 43 cases, no cross-reaction of the applied antibodies (C1P83, Ra96, CA19-9, 17-1A, C54-0). Two patients with chronic pancreatitis were positive for C54-0 or C1P83. A third patient with chronic hepatitis C was positive for C54-0.



ure 1. The percentage of positive samples for each monoclonal ibody and the combined evaluation in the bone marrow and the itoneal cavity of (a) colorectal and (b) gastric cancer patients.

olorectal Cancer

In 49% (53 of 109) of the patients, stained cells were ected in the bone marrow (33% [35/105]) or in the itoneal cavity (31% [34/109]). Fourteen patients showed sitive staining in both compartments.

Kl-1 showed the highest detection rate in bone marrow nples, followed by CA19-9 and C1P83 (anti-CEA). In periieal cavity samples, CA19-9 and C1P83 reacted with the hest frequency with tumor cells. The combination of all ibodies significantly increased the detection rate (Fig. 1A).

In bone marrow and peritoneal cavity samples, the detecn rate increased in parallel with the tumor stage (Fig. 2). erestingly, already 19% of patients with a stage I tumor l positive cells within the peritoneal cavity.

Forty-four percent of R0-resected patients (n = 96) owed tumor cell spread either in the peritoneal cavity !% [21/94]) or in the bone marrow (33% [27/83]).

The postoperative 4-year survival rate was determined in) patients. The cumulative survival rates for peritoneal ity and bone marrow findings are shown in Figure 3. ter 4 years, 28% of patients with positive immunocytoic findings in the peritoneal cavity were alive *versus* 60% patients with negative findings (p = 0.0079). No correon was found by evaluating the results of bone marrow nples.

To determine if the antibodies vary in their correlation with the survival rate, each single antibody result was evaluated separately. A highly significant correlation with the survival and peritoneal cavity findings was found for C1P83 ($p < 0.0001$) and CA19-9 ($p = 0.0002$). The findings with C54-0 ($p = 0.015$) and Ra96 ($p = 0.03$) were also statistically significant. Only 17-1A did not detect prognostically relevant cells ($p = 0.48$). In bone marrow samples, Ra96-positive patients showed a worse but statistically not significant ($p = 0.2$) prognosis: all 9 positive patients died within 3 years, compared to a 40% survival rate of negative patients. All other antibodies, including Kl-1, showed no prognostic significance (C1P83: $p = 0.7$; CA19-9: $p = 0.48$; 17-1A: $p = 0.9$; Kl-1: $p = 0.42$).

Consequently, further evaluation of peritoneal cavity samples excluded 17-1A findings. Additionally, the results from C54-0 were not taken into account because this antibody did not show prognostic significance in gastric cancer and reacted with normal cells of lavage samples from three cases in the control group.

Patients were evaluated according to their tumor stage to determine if the occurrence of isolated tumor cells has a prognostic significance independent from the UICC classification. Table 1 summarizes the results for each tumor stage. In all stages, patients with positive peritoneal cavity findings showed a worse survival rate than negative patients (stage I: 75% *vs.* 93%; stage II: 80% *vs.* 82%; stage III: 33% *vs.* 65%; stage IV: 8% *vs.* 27%).

The low number of cases hindered the Kaplan-Meier calculation for each stage. Therefore, patients with an "early tumor stage" (stages I and II, n = 56) and advanced

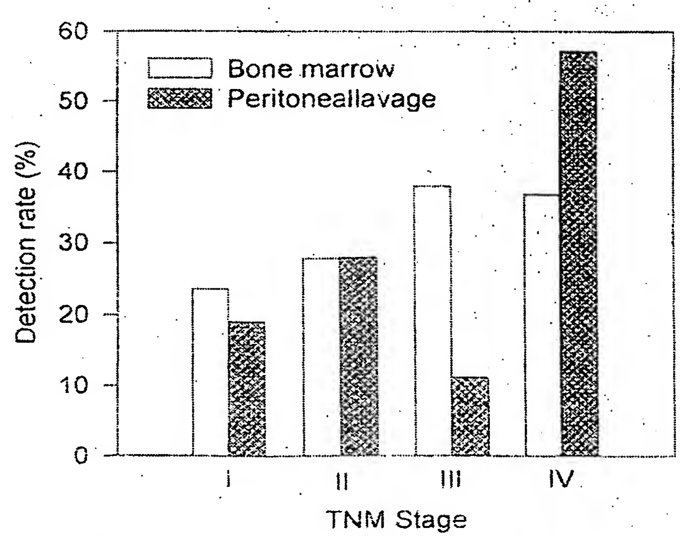
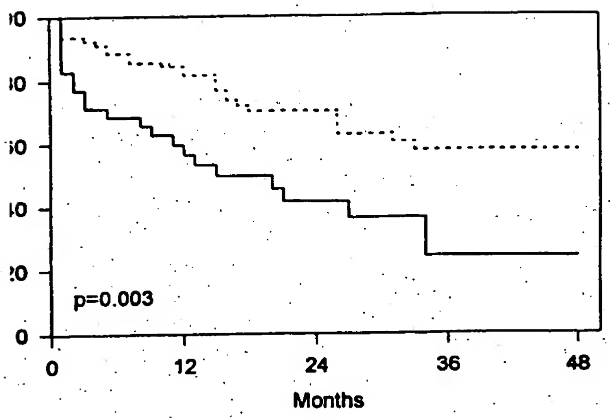
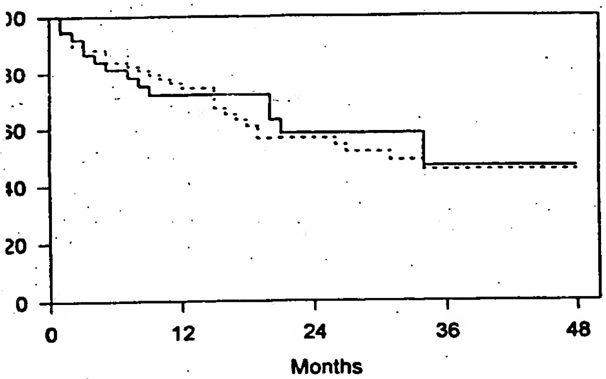


Figure 2. Correlation between the UICC tumor stage and positive findings in the bone marrow (bm) and peritoneal cavity (pc) of colorectal cancer patients. Bone marrow: stage I, n = 15; stage II, n = 33; stage III, n = 28; stage IV, n = 29. Peritoneal cavity: stage I, n = 21; stage II, n = 33; stage III, n = 27; stage IV, n = 28.





3. Kaplan-Meier calculation for the cumulative 4-year survival patients with colorectal cancer with antibody-positive (Ab-posi- ersus antibody-negative (Ab-negative) samples. (a) Results of eal lavage samples (p = 0.0079). — Ab-positive (n = 34); -negative (n = 75) (b) Evaluation of bone marrow samples (p = — Ab-positive (n = 35); - - - Ab-negative (n = 70)

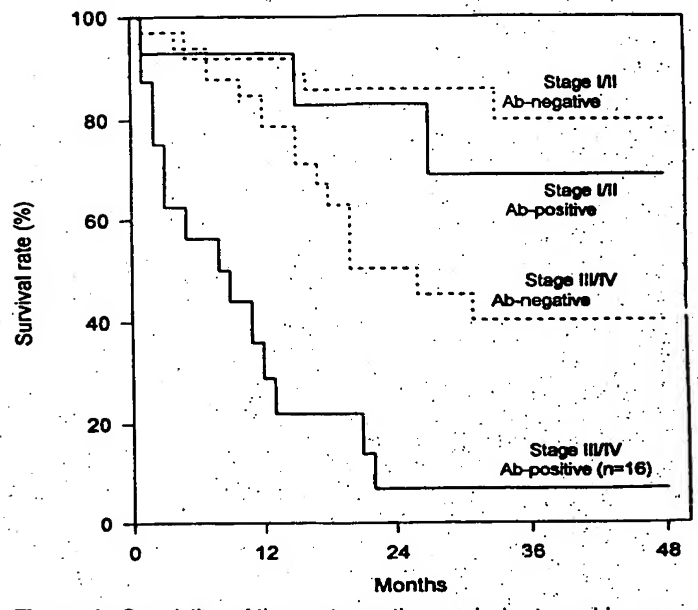


Figure 4. Correlation of the postoperative survival rate and immunocytologic findings in the peritoneal cavity of patients with colorectal cancer (17-1A and C54-0 antibody results are excluded). Cases are compared with early tumor (stages I and II) (p = 0.48) and advanced (stages III and IV) (p < 0.0001). Stage I/II Ab-negative (n = 42); Stage I/II Ab-positive (n = 14); Stage III/IV Ab-negative (n = 34)

Table 1. SURVIVAL OF COLORECTAL CANCER PATIENTS WITH POSITIVE/NEGATIVE FINDINGS IN THE PERITONEAL LAVAGE (PL) AND IN THE BONE MARROW (BM)

Positive PL	Negative PL	Positive BM	Negative BM
3/4 (75)	13/14 (93)	4/4 (100)	11/12 (92)
8/10 (80)	23/28 (82)	7/8 (87)	18/24 (75)
1/3 (33)	15/23 (65)	9/10 (90)	9/20 (45)
1/13 (8)	3/11 (27)	0/9 (0)	5/17 (29)

are the numbers of patients that survived within the observation period in to the total number of cases with tumor stages I, II, III, and IV; the value in eses is the percentage of 4-year survival. (17-1A and C54-0 antibody are excluded)

cancer (stages III and IV, n = 50) were combined and evaluated with the log-rank test.

The survival rates of patients with stage I and II disease and a positive or negative peritoneal cavity finding ran parallel in the first 2 postoperative years. Afterward, the curves divided: after 3 years, 80% of patients with negative results were still alive *versus* 69% in the positive group ($p = 0.48$). In patients with stage III and IV disease, the difference between positive and negative findings became statistically highly significant ($p < 0.0001$) (Fig. 4). In bone marrow samples, no correlation was seen by evaluation of single tumor stages except in stage IV patients (see Table 1) and by Kaplan-Meier analysis of early and advanced patients (data not shown).

Gastric Cancer

Positive stained cells were detected in 64% (54 of 84) of the patients, with 53% (33 of 62) positive peritoneal cavity samples and 48% (30 of 62) positive bone marrow samples. Nineteen patients had disseminated tumor cells in both compartments. Figure 5 shows a typical microscopic picture of a C1P83-positive peritoneal cavity sample.

KI-1 showed the highest detection rate in bone marrow samples, followed by C1P83 and CA19-9. In peritoneal cavity samples, C1P83 and CA19-9 stained tumor cells with the highest frequency. The combination of all antibodies significantly increased the detection rate (Fig. 1B).

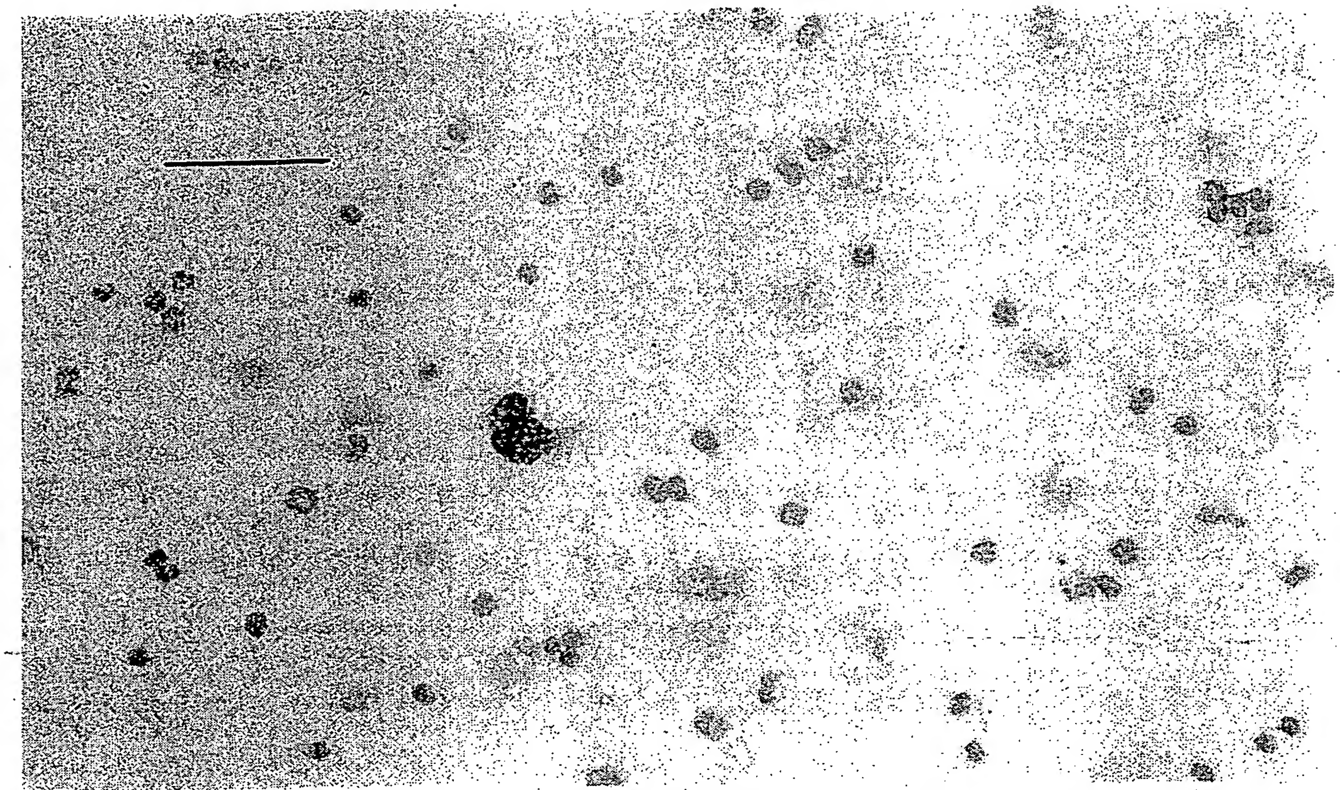
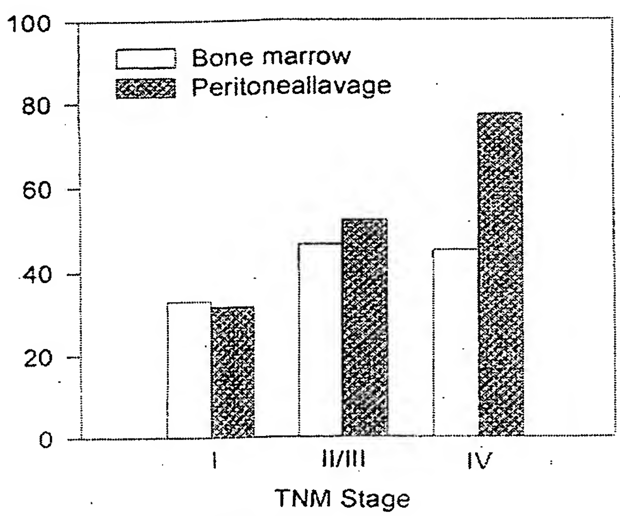


Figure 5. Cytospin of a peritoneal lavage sample from a patient with gastric cancer (stage II) stained with C1P83 (anti-CEA) antibody. Bar = 40 μm.

In peritoneal cavity samples, the detection rate increased parallel with the tumor stage (Fig. 6), showing positive nples in 32% of stage I and 77% of stage IV patients.



ıure 6. Correlation between the UICC tumor stage and positive lings in the bone marrow and peritoneal cavity of gastric cancer ients. Bone marrow: stage I, n = 20; stages II and III, n = 23 (stage ı = 9; stage III, n = 14); stage IV, n = 19. Peritoneal cavity: stage I, 19; stages II and III, n = 21 (stage II, n = 10; stage III, n = 11); stage n = 22.

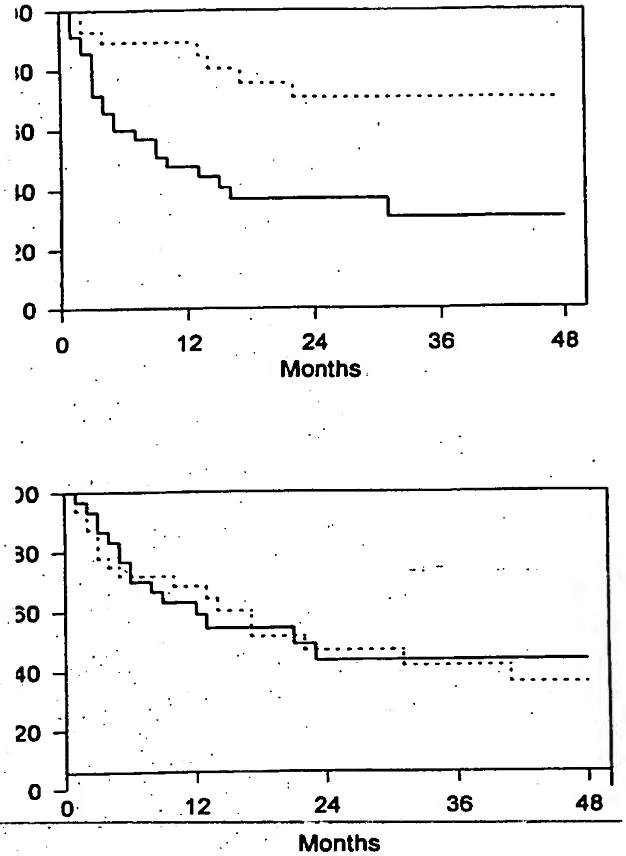
Only a small increase was seen between bone marrow findings of progressing tumors.

R0-resected patients showed a tumor cell spread in 55% of the peritoneal lavage (n = 31) and in 49% of the bone marrow probes (n = 41).

The cumulative postoperative survival rate was correlated with the immunocytologic findings and showed highly significant results in peritoneal cavity samples ($p = 0.0038$), but no correlation was seen in bone marrow samples ($p = .1$) (Fig. 7).

The single antibody evaluation showed highly significant results for Ra96, CA19-9, and C1P83 ($p < 0.0001$) in peritoneal cavity samples. The 17-1A antibody also correlated ($p = 0.023$), but no correlation was found with C54-0 ($p = 0.9$). In bone marrow samples, C1P83 and Kl-1 detected prognostically relevant cells. All 14 patients with C1P83 tumor cell staining died within 2 years ($p = 0.08$). In patients with advanced cancer, Kl-1 detection of tumor cells correlated with a worse survival ($p = 0.12$).

Patients were evaluated according to tumor stage to determine if the occurrence of isolated tumor cells had a prognostic significance independent of the UICC classification. Results from C54-0 and 17-1A were excluded. Table 2 summarizes the results for each tumor stage. In all stages except stage I, patients with positive peritoneal findings showed a worse survival rate than negative patients (stage II: 50% *vs.* 100%; stage III: 40% *vs.* 56%;



ı 7. Kaplan-Meier calculation for the cumulative 4-year survival gastric cancer patients with antibody-positive (Ab-positive) *versus* tibody-negative (Ab-negative) samples. (a) Results of peritoneal samples (p = 0.0038). —— Ab-positive (n = 33); - - - Ab-nega- ı = 29) (b) Evaluation of bone marrow samples (p = 1). ɔ-positive (n = 30); - - - Ab-negative (n = 32)

IV: 7% *vs.* 50%). In stage I, the group size was too to draw any conclusions (2 positive patients *vs.* 18 ive).

plan-Meier curves were calculated with the log-rank y combining stage I and II patients and stage III and IV ıts. As in colorectal cancer, the survival curves in stage II patients ran parallel for 2 years before dividing. 4 years, patients with negative peritoneal samples ed a significantly better survival rate than patients with ve findings (80% *vs.* 54%, p = 0.02). This difference ven more striking in advanced patients: The Kaplan- r calculation showed that all positive patients die n 2.5 years, but 54% of negative patients survive at 4 years (p = 0.02) (see Fig. 8).

bone marrow samples, patients with stage III and IV ed a worse survival when tumor cells were detected: at ırs, 14% of stage III and 0% of stage IV positive ıts survived *versus* 50% and 30% of negative patients, ctively. The combined evaluation of stage I and II and

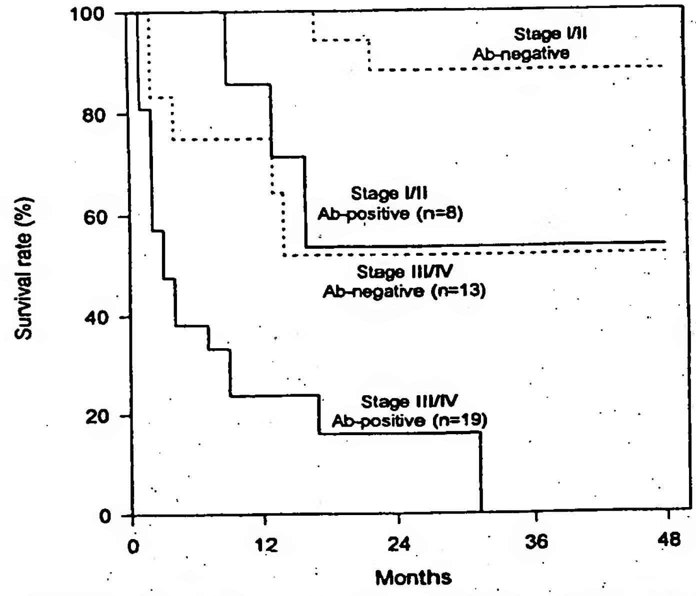


Figure 8. Correlation of the postoperative survival rate and immunocytologic findings in the peritoneal cavity of patients with gastric cancer (17-1A and C54-0 antibody results are excluded). Cases are compared with early tumor (stages I and II) (p = 0.02) and advanced (stages III and IV) (p = 0.02). Results with the antibodies 17-1A and C54-0 were excluded. Stage I/II, Ab-negative (n = 22)

stage III and IV patients, as performed by Kaplan-Meier analysis, showed no statistically significant differences in either group.

DISCUSSION

By immunocytologic techniques, it has become possible to detect isolated tumor cells in the bone marrow of various cancer patients. Most studies have been performed with breast cancer patients using an antibody specific for epithe-

Table 2. SURVIVAL OF GASTRIC CANCER PATIENTS WITH POSITIVE/NEGATIVE IMMUNOCYTOLOGICAL FINDINGS IN THE PERITONEAL LAVAGE (PL) AND IN THE BONE MARROW (BM)

Stage	Positive PL	Negative PL	Positive BM	Negative BM
I	2/2 (100)	15/18 (83)	7/7 (100)	6/9 (67)
II	3/6 (50)	4/4 (100)	4/4 (100)	2/3 (67)
III	2/5 (40)	5/9 (56)	1/7 (14)	5/10 (50)
IV	1/14 (7)	2/4 (50)	0/8 (0)	3/10 (30)

Values are the numbers of patients that survived within the observation period in relation to the total number of cases with tumor stages I, II, III, and the IV; value in parentheses is the percentage of 4-year survival. (17-1A and C54-0 antibody results are excluded)

l cells to detect tumor cell spread in the bone marrow at : time of operation.[18] A strong correlation between tumor ll detection and survival could be seen; hence, in these tients, the finding of isolated cancer cells may serve as a w prognostic marker.[4] Further studies were published scribing a similar approach to search for isolated tumor lls in the bone marrow of patients with lung cancer,[5] ostatic cancer,[7] and neuroblastoma.[6]

In contrast to these malignancies, bone metastases are e in gastric and colorectal cancer. However, peritoneal rcinosis or metastases within the peritoneal cavity (e.g., in : liver and lymph nodes) occur in 90% of the patients.[11] ierefore, we investigated peritoneal cavity samples from lorectal and gastric cancer patients by an immunocyto- gic approach using a panel of five antibodies that react th several tumor-associated antigens. We also investi- ted the bone marrow because it might serve as a filtering stem of the bloodstream, so circulating tumor cells could come detectable due to a cumulative effect.[9]

Previously,[10] we showed that our approach allows highly ecific tumor cell detection in the bone marrow and in the ritoneal cavity. Despite the theoretical risk of nonspecific uning, we found no positive cells in the samples from the ntrol group. In this study, we confirmed the practicability our definition of tumor cell positivity (the finding of only e stained cell with one monoclonal antibody). The en- ged control group contained only two positive bone mar- w and three positive peritoneal lavage samples. Two tients were treated by a Whipple operation because of ong suspicion of pancreatic cancer. A third patient suf- ed from chronic hepatitis C and liver cirrhosis and owed positive lavage cells for C54-0. Especially in peri- ieal lavage samples, there is a theoretical risk of nonspe- ic staining of mesothelial cells in the peritoneal cavity.[19] ierefore, we propose excluding C54-0 from peritoneal age examination. However, it will be interesting to fol- w up the three patients with positive samples because of : remaining uncertainty about the diagnosis.

In our study, we found disseminated tumor cells in the ne marrow and the peritoneal cavity with similar fre- ency. Even in stage I, when direct tumor access can be mpletely excluded, about 22% of colorectal and 30% of stric cancer patients had circulating tumor cells within the ritoneal cavity. This observation supports data from a tologic study in gastric cancer.[20] In this study 3% of stage ;astric cancer patients had viable tumor cells within the ritoneal cavity; they most likely reached the peritoneum pores and lymph vessels. In accordance with the signif- intly higher sensitivity of the immunocytologic method,[3] : achieved higher detection rates. Therefore, we found ong evidence that tumor cell spread is a general feature of strointestinal cancers and must be classified in most pa- nts as generalized disease.

Whether isolated tumor cells can form metastatic disease d are therefore of prognostic significance remains to be icidated. In our study, colorectal cancer patients whose bone marrow samples showed tumor cells—stained with Ra96 and C1P83 (anti-CEA)—had a worse prognosis. The anticytokeratin antibody KI-1 detected a high-risk group of gastric cancer patients who suffered from stage III or IV disease. These results support to some extent data from Lindemann et al.[21] Using an anticytokeratin antibody, they found high numbers of tumor cells in the bone marrow of colorectal cancer patients and suggested that these cells had independent prognostic significance.

Our results strongly indicate that in gastrointestinal cancer, the investigation of peritoneal cavity cells is more relevant than the bone marrow approach. This finding is in accordance with the rarity of bone metastases but the high frequency of metastases within the peritoneal cavity.[11]

In colorectal and gastric cancers, the cumulative survival rates significantly correlated with the immunocytologic findings. Furthermore, patients with small tumors (stage I or II) and positive peritoneal cavity samples showed a worse prognosis; in gastric cancer, the difference was statistically significant ($p = 0.02$). In the advanced tumor stage (stages III and IV), all gastric cancer patients with positive staining are supposed to die within 2.5 years, but 54% of patients with negative staining survived. In colorectal cancer, all but 2 patients in the stage III and IV group with positive findings died, in contrast to a 40% 4-year survival rate in the negative group. Although no correlation between immunocytologic findings and the single tumor stage could be statistically calculated in most cases, striking differences could be seen in colorectal cancer stage I and IV patients. In stage I, 1 of 4 positive patients but only 1 of 14 negative patients died within 4 years. In stage IV, almost all positive patients (12 of 13) died within 2 years, but 27% of the 11 negative patients survived 4 years.

These results strongly suggest that the immunocytologic staining of peritoneal lavage samples serves as a new prognostic marker. A recently published method allows much more rapid immunostaining of peritoneal cavity samples by reducing the antibody incubation time with a microwave irradiation of the cells.[22] By using such a technique, it might be possible to get a diagnostic result while the operation is still in progress. This could help to guide surgical and adjuvant therapy. Stage IV patients with a negative peritoneal lavage might benefit from a radical surgical approach to achieve a R0 resection, but stage IV patients with a positive lavage most likely would not benefit from this approach. In gastric cancer patients, intraoperative prophylactic application of carbon-adsorbed mitomycin was shown to reduce the rate of tumor relapse.[1] This treatment could be helpful in lavage-positive patients with early tumor stages. Furthermore, it might be useful to offer adjuvant therapy in general to patients with positive peritoneal findings and a tumor stage I or II. Currently, these patients are excluded from adjuvant treatment because of the overall low risk of cancer relapse.[2]

It is unclear why the prognostic significance of bone marrow cells is of lower value. Perhaps the contact of tumor cells with peritoneal cells supports their ability to develop the full meta-

›henotype, a hypothesis supported by the clinical obser- of a high peritoneal carcinosis rate in colorectal and ılly gastric cancer.[11] Cells in the bone marrow may be in /rong'' environment and be kept in a dormant state, as om Pantel et al.[23] suggest. Further studies will focus on terizing the isolated cancer cells to elucidate local fac- ıt may be important in the progress to metastatic disease.

present immunocytologic approach has the disadvan- f being time-consuming and dependent on the skill of /estigator. Thus, from a practical point of view, this ıch is unsuitable as a widely used routine method. :ore, the nested reverse transcriptase–polymerase reaction (RT-PCR) has been used to detect dissemi- tumor cells. Recently, it was shown that a nested :R with cytokeratin CK20 enables specific detection ıor cells in the bone marrow of patients with gastro- nal cancer.[24] This approach is not practicable in the ıeal cavity. The nested RT-PCR with CEA, which has y been shown to detect gastrointestinal cancer cells in narrow samples,[25] might also be suitable for perito- ıvage samples. Although nested RT-PCR is a useful :s reliability remains to be proven in the diagnosis of intestinal cancer dissemination. The standardization ; extremely sensitive technique is one of the main les and is currently being investigated.[26]

ummary, by using an immunocytologic approach, we d for the first time that a minimal residual disease ›ecomes detectable in the peritoneal cavity of patients :olorectal and gastric cancer. The occurrence of iso- umor cells could be correlated with a worse progno- en in early cancer stages. Therefore, the detection of :ells can serve as a new prognostic marker and will › guide adjuvant therapy.

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APPENDIX F

Differential Frequencies of $p16^{INK4a}$ Promoter Hypermethylation, *p53* Mutation, and K-*ras* Mutation in Exfoliative Material Mark the Development of Lung Cancer in Symptomatic Chronic Smokers

By M. Kersting, C. Friedl, A. Kraus, M. Behn, W. Pankow, and M. Schuermann

Purpose: The aim of this study was to investigate the frequency of three (epi)genetic alterations (*p53* and K-*ras* mutations and $p16^{INK4a}$ promoter hypermethylation) in symptomatic chronic smokers compared with patients with lung cancer and to evaluate the use of exfoliative material for such analyses.

Patients and Methods: Fifty-one patients with histologically confirmed lung cancer and 25 chronic smokers (> 20 pack-years) were investigated for mutations in the K-*ras* (codon 12) and *p53* (codons 248, 249, and 273) genes and for allelic hypermethylation of the $p16^{INK4a}$ gene. DNA was isolated from sputum and bilateral bronchial lavage, and brushings were taken at bronchoscopy.

Results: Forty-one genetic lesions were detected within exfoliative material from the group of 51 patients with lung cancer and 10 lesions in the chronic smoker group. K-*ras* mutations occurred exclusively in the lung cancer group, whereas *p53* mutations and $p16^{INK4a}$ promoter hypermethylation were also found in chronic smokers. Three of eight chronic smokers who harbored an (epi)genetic alteration were subsequently diagnosed with lung cancer. Analysis of sputum yielded information equivalent to that of samples obtained during bronchoscopy.

Conclusion: $p16^{INK4a}$ promoter hypermethylation and *p53* mutations can occur in chronic smokers before any clinical evidence of neoplasia and may be indicative of an increased risk of developing lung cancer or of early disease. K-*ras* mutations occur exclusively in the presence of clinically detectable neoplastic transformation. Molecular analysis of sputum for such markers may provide an effective means of screening chronic smokers to enable earlier detection and therapeutic intervention of lung cancer.

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THE ACCUMULATION of genetic damage is a hallmark in the development of lung cancer, a tumor with growing incidence and the leading cause of cancer-related deaths in industrial nations. The vast majority of lung cancers are smoking-related, whereby the number of genetic lesions present correlates with the intensity of carcinogen inhalation. Despite intensive clinical research, there has been no significant improvement in the therapy of lung cancer during the past 10 years. This is largely a result of the early metastatic spread of such tumors relative to the time of clinical diagnosis. Using the current standard methods of diagnosis and treatment, fewer than 15% of patients with lung cancer will survive their disease.[1] The development of efficient diagnostic methods to enable earlier detection and therapeutic intervention in lung cancer is clearly important.

In one recent study, screening using cytologic examination of sputum identified a group of patients with preinvasive and microinvasive lung cancer, who demonstrated high survival rates after surgical removal or localized therapy.[2] In other studies, however, annual cytologic sputum examination combined with chest radiography did not improve the overall survival of patients,[3,4] probably because microscopic metastatic disease had already occurred.

Significant progress has now been made in the understanding of the genetic basis of lung cancer,[5,6] which raises the hope that molecular markers could be used to detect lung cancer earlier in its natural history. To date, molecular analyses of sputum or bronchial lavage fluid have concentrated on mutations in oncogenes, such as K-*ras*, or tumor suppressor genes, such as *p53*, as potential markers of lung cancer[7] (reviews[8-11]). Mutations in K-*ras* are found in non–small-cell lung cancer (NSCLC), predominantly in adenocarcinoma, and the rate ranges between 15% and 50%, depending on the study material and the sensitivity of the assay used.[12-18] The vast majority of K-*ras* mutations affect codon 12 (> 90%).[19] Mutations of the *p53* gene are detectable in 50% to 70% of patients with lung cancer and are found in all histologic types.[20-24] These mutations

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Table 1. Frequency of *p53* (Codon 248, 249, and 273) and K-*ras* (Codon 12) Mutations and $p16^{INK4a}$ Promoter Hypermethylation Status in Exfoliative Material (Sputum, Bronchial Lavage, and Brushings) From Patients With Histologically Confirmed Lung Cancer and From Symptomatic Chronic Smokers

	Total No. of Patients	*p53* Mutation		K-*ras* Mutation		$p16^{INK4a}$ Hypermethylation		Any Lesion	
		No.	%	No.	%	No.	%	No.	%
Total patients with tumors	51	7	14	8	16	26	51	35	69
Tumor cell type									
NSCLC	31	4	13	8	26	18	58	25	81
Squamous cell	22	3	14	4	18	13	59	17	77
Adenocarcinoma	5	0		1	20	3	60	4	80
Large cell	2	1	50	2	100	1	50	2	100
Other	2	0		1	50	1	50	2	100
SCLC	20	3	15	0		8	40	10	50
Chronic smokers	25	3	12	0		7	28	8	32

comprise both allelic loss and point mutations, the latter clustering within the hot spot regions of the *p53* gene.[25] Although K-*ras* mutations seem to occur late in lung cancer tumorigenesis,[26] somatic alterations of the *p53* gene are found in different tumor stages and may even occur in metaplastic and dysplastic states.[27-29] More recently, inactivation of the $p16^{INK4a}$ tumor suppressor gene has been shown to occur commonly in lung cancer,[30-32] resulting from either allelic loss or mutation of $p16^{INK4a}$ or regional hypermethylation of CpG islands in its promoter region.[33] In contrast, $p16^{INK4a}$ promoter hypermethylation is detectable neither in normal lung tissue nor in a variety of other types of non-neoplastic tissue investigated.[30,34] Little is known, however, about the sequence of these various events in the development of lung cancer.

In the present study, we examined exfoliative material (sputum, bronchial lavage fluid and brush cytology) from 51 patients with lung cancer and 25 chronic smokers (> 20 pack-years and suffering from benign lung disease) for the prevalence of CpG-island hypermethylation of $p16^{INK4a}$ and mutations in the K-*ras* and *p53* genes. Our aim was to establish the frequency of each of these genetic or epigenetic lesions in chronic smokers compared with patients with established lung cancer and, thereby, to investigate their potential benefit as molecular markers of preneoplastic or early disease. In particular, we addressed the question of whether such genetic and epigenetic changes can be detected in easily obtainable exfoliative material such as sputum, which would render such analyses more suitable for a screening program.

PATIENTS AND METHODS

Patient Selection

All diagnostic bronchoscopies were performed in the Department of Respiratory Medicine in the Krankenhaus Neukölln in Berlin between February 1996 and February 1999 by one of the authors (W.P.). Patients were selected at random during the author's (W.P.) duty periods in the Endoscopy Unit. Patients were selected for the lung cancer group if radiography was suggestive of an endoscopically visible tumor. Symptomatic chronic smokers were included in the study only when chest radiography was not indicative of tumor or chronic inflammatory disease such as sarcoidosis. All were heavy smokers of greater than 20 pack-years. All subjects gave written informed consent before the investigation.

Induced sputum was obtained during local anesthesia using 10 to 15 mL of nebulized lignocaine. Flexible fiberoptic bronchoscopy was performed using an Olympus fiberoptic bronchoscope (Olympus Optical Co, Hamburg, Germany) via the oral route. Intrabronchial procedures were performed in the following sequence: bronchial lavage with 10 mL of sterile saline, brushings from the main bronchus or tumor site, then biopsy of the tumor, bilaterally, starting with the nontumor side. In the chronic smokers, lavage and brushings were performed in the main bronchi in each lung and one to two bronchial biopsies were taken from one side. Brushings were suspended in sterile saline. Biopsies were fixed in 4% formalin. Sputum and bronchial lavage specimens were each mixed with 100 mg of acetylcysteine for 10 minutes to break down mucus, before passing the fluid through several layers of sterile gauze. After centrifugation (2000 rpm for 5 minutes at 4°C), the cell pellet was washed once in phosphate-buffered saline. Cell pellets were stored in microtubes at −20°C.

The mean age of the lung cancer patient group was 63 years (range, 46 to 84 years; median, 62 years) and included male (n = 39) and female subjects (n = 12). The mean age of the symptomatic chronic smoker group was 56 years (range, 38 to 79 years; median, 54 years) and included male (n = 22) and female subjects (n = 3).

Patients were clinically staged by the author (W.P.), and tumor biopsies were independently graded by two pathologists. Of 51 patients with lung cancer, 20 had small-cell lung cancer (SCLC) and 31 had NSCLC; histologies are listed in Table 1.

Table 2 shows the clinical staging of the patients selected for the study. Five patients had stage I disease, four had stage II disease, 20 had stage III disease, and 22 had stage IV disease, according to the revised staging system.[35]

Tumor Biopsies

DNA from paraffin-embedded tumor biopsy tissue was extracted from 10-μm sections according to "Method A," as described by Frank et al.[36] In parallel, sections were hematoxylin and eosin–stained and pathologically reviewed to ensure inclusion of tumor tissue.

Table 2. Frequency of Each Genetic Lesion Detected in Relation to the Tumor Staging

		p53 Mutation		K-ras Mutation		$p16^{INK4a}$ Hypermethylation	
	Total No.	No.	%	No.	%	No.	%
Pathologic stage							
IB	5	0		0		3	60
IIB	4	0		1	25	4	100
IIIA	6	1	17	0		4	67
IIIB	14	0		3	21	5	36
IV	22	6	27	4	18	10	45

Sputum and Bronchial Lavage Samples

DNA was extracted from 50% of homogenized pellets of sputum, bronchial lavage fluid (BAL), or brushings, using a Qiagen Tissue Kit (Qiagen, Hilden, Germany) for DNA preparation, according to the suppliers' specifications. Each DNA sample was tested for the presence of genomic $p16^{INK4a}$ alleles by polymerase chain reaction (PCR) using primer pair p16-W.[37]

p53 *Mutation and K*-ras *Mutation Analysis*

Mutation analysis was performed as previously described.[7] Briefly, 50 to 200 ng of DNA were preamplified for 30 cycles in a volume of 50 μL containing 1 U of eLONGase (GibcoBRL, Life Technologies, Rockville, MD). Purified PCR products were diluted 1:30 and subjected to the following PCR–restriction fragment length polymorphism analysis. Two microliters of the diluted PCR products was reamplified with mismatch primers and digested with appropriate endonucleases. Codons 248, 249, and 273 were examined for *p53* mutations and codon 12 for K-*ras* mutation. Primer sequences and reaction conditions have been described elsewhere.[7] A patient was considered to be a carrier of a mutation when at least one sample (sputum, side-specific BAL or side-specific brushings) revealed a clear positive result in PCR analysis.

$p16^{INK4a}$ *Hypermethylation Assay*

Bisulfite conversion. Existing bisulfite conversion protocols[37-39] were modified for use with this patient material. Briefly, 10% of prepared DNA (10 to 200 ng) and 2 μg of Poly(dA-dT)Poly(dA-dT) copolymers (Amersham Pharmacia Biotech, Bucks, United Kingdom) were denatured for 20 minutes at 42°C by adding 1 N NaOH in a volume of 50 μL (to a final concentration of 0.3 mol/L). Fresh solutions of 10 mmol/L hydroquinone (Sigma Chemical Co, St Louis, MO; 30 μL) and 3 mol/L sodium bisulfite, pH 5.0 (Sigma Chemical Co; 520 μL) were added and the solution was gently mixed, overlayed with mineral oil, and incubated in the dark for 12 to 13 hours at 50°C. The aqueous phase was recovered using the Wizard DNA Clean-up System (Promega, Madison, WI). The elution efficiency of small DNA quantities was significantly improved by successive elution of bound DNA using prewarmed (80°C) TE buffer, pH 7.6 (50 μL of TE, 15 minutes incubation; 30 μL TE, 1 minute; centrifugation at 9,000 rpm for 20 seconds). The purified DNA was subsequently mixed with 1 mol/L NaOH to a final concentration of 0.3 mol/L and incubated for 20 minutes at 37°C to ensure complete desulfonization. DNA was ethanol-precipitated in the presence of 10% volume sodium acetate, and the resulting pellet, after washing with 70% ethanol, was resuspended in 50 μL of H_2O and stored at −20°C.

Primer extension preamplification (PEP). The genomic amplification was performed following a protocol by Zhang et al.[40] Twenty microliters of bisulfite-treated DNA was added to 5 μL of a 400-μmol/L solution of gel-filtrated N15 primers (TIB MOLBIOL, Berlin, Germany), 0.6 μL of dNTPs (each at 20 mmol/L), 6 μL of 10× PCR buffer, and 5 U of *Taq* polymerase (Qiagen) and H_2O (DuPont Merck Pharmaceutical Company, Wilmington, DE) to yield a total volume of 60 μL (whereby the quality of random primer synthesis proved to be of special importance). After initial denaturation at 94°C for 3 minutes, 50 primer extension cycles were performed in a Perkin Elmer 9,600 thermocycler (Perkin Elmer, Norwalk, CT) using the following parameters: 1-minute denaturation at 92°C, 2-minute annealing at 37°C, followed by a 3-minute linear ramping to 55°C, and 4-minute elongation at 55°C. Protocols with a faster ramping rate or stepwise temperature progression resulted in inefficient PEP amplification.

Semi-nested methyl-specific PCR (MSP). The detection of hypermethylated $p16^{INK4a}$ alleles was facilitated using a semi-nested PCR protocol in a 25-μL reaction volume. In the first PCR, a 5-μL aliquot of the PEP product was added to 200 μmol/L dNTPs, 0.4 μmol/L each primer (p16Mf: 5'-TTA TTA GAG GGT GGG GCG GAT CG; p16M2r 5'-CCA CCT AAA TCG ACC TCC GAC CG), 1× PCR buffer (Qiagen) and 0.65 U of *Taq* Polymerase. The second PCR contained 5 μL of the 1:10 diluted product of the first PCR, 200 μmol/L of dNTPs, 0.4 μmol/L of each primer (p16Mf, p16Mr 5'-GAC CCC GAA CCG CGA CCG TAA), 1× PCR buffer (Qiagen) and 0.65 U of *Taq* Polymerase. Both reactions comprised 30 cycles with 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 40 seconds, with initial denaturation at 95°C for 3 minutes and a final elongation at 72°C for 10 minutes.

To control for complete bisulfite conversion and subsequent PEP amplification, a semi-nested PCR was also performed for the non-methylated alleles. The same protocol, including 1× solution Q (Qiagen) to optimize buffer conditions, was performed using primers p16Uf (5'-TTATTA GAGGG TGGGG TGGAT TG) and p16U2r (5'-CCACC TAAAT CAACC TCCAA C) in the first reaction and primers p16Uf and p16Ur (5'-CAACC CCAAA CCACA ACCAT AA) in the second. The annealing temperature was lowered to 58°C.

Statistics

Statistical analysis of frequency distributions was evaluated by the χ^2 or Fisher's exact test, as appropriate, using the Statistical Package

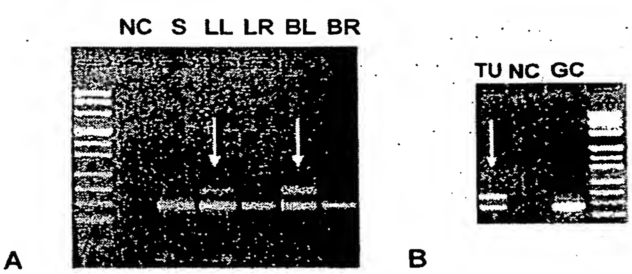

Fig 1. K-*ras* analysis. (A) Sputum, bronchial lavage left and right, brushings left and right, negative control, demonstrating mutations in lavage and brushings from the left side (adenocarcinoma left lobe). (B) Mutation confirmed in tumor biopsy. Abbreviations: S, sputum; LL, lavage left; LR, lavage right; BL, brushings left; BR, brushings right; NC, negative control; TU, tumor biopsy; GC, genomic DNA.

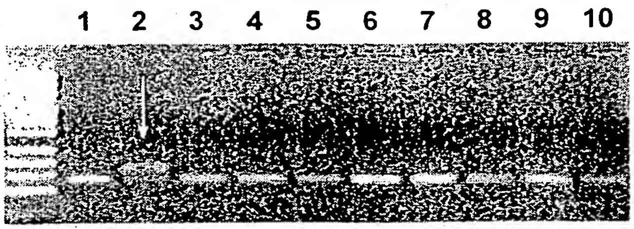

Fig 2. Example of *p53* (codon 273) restriction fragment length polymorphism analysis of bronchial lavage from patients with lung cancer. The arrow in lane 2 indicates a mutation.

for Social Science (SPSS, Inc, Chicago, IL). Statistical significance was taken as $P < .05$.

RESULTS

p53 mutations (codon 248, 249, or 273) were detected in seven samples (14%) from patients with tumors, of whom three had squamous cell carcinoma (14%; n = 22), one had large-cell carcinoma (50%; n = 2), and three had SCLC (12%; n = 20) (Table 1). Mutation in the *p53* gene was also detected in three samples (12%) from chronic smokers at the time of bronchoscopy. There was no statistically significant difference between patients with lung cancer and chronic smokers in the frequency of *p53* mutation (P = .572).

Mutations in the K-*ras* gene (codon 12) were found in eight samples (16%) from patients with confirmed lung cancer, including four patients with squamous cell carcinoma (18%; n = 22), one with adenocarcinoma (33%; n = 3), and two with large-cell carcinoma (100%; n = 2) (Table 1). No K-*ras* mutation was found in any patient with SCLC or in any of the chronic smokers. There was a statistically significant difference between patients with lung cancer and chronic smokers in the frequency of K-*ras* mutation (P = .034). Figures 1 to 3 show examples of samples investigated for K-*ras* and *p53* mutations and $p16^{INK4a}$ hypermethylation.

Modification of the standard MSP protocol[37,41] was necessary to analyze the exfoliative material for $p16^{INK4a}$ methylation status (see Methods). This was achieved by inserting a genomic amplification step, as described by Zhang et al,[40] before performing a semi-nested, rather than a conventional, MSP analysis. Figure 4 illustrates the gain in sensitivity achieved by the modified MSP protocol (Fig 4A) compared with the conventional protocol (Fig 4B).

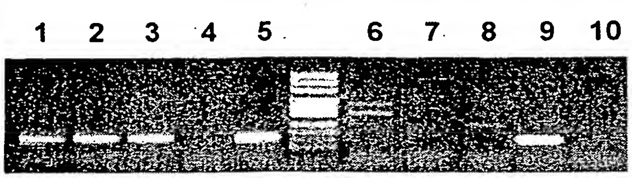

Fig 3. $p16^{INK4a}$ promotor hypermethylation analysis of sputum-DNA from lung cancer patients. Lanes 1 to 3, 5, and 9 represent hypermethylated alleles.

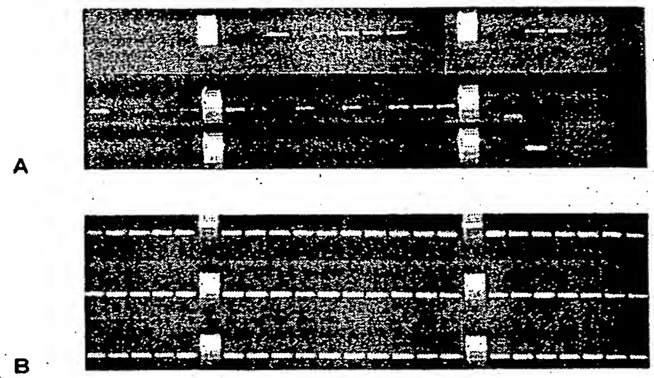

Fig 4. Control of sputum-derived DNA for bisulfite conversion with primers specific for nonmethylated sequences. (A) Conventional methyl-specific PCR (MSP) and (B) modified protocol with genomic amplification (PEP) and subsequent semi-nested PCR (see Patients and Methods).

Hypermethylation of the $p16^{INK4a}$ promoter was found in 26 patients with lung cancer (51%; n = 51) and in seven chronic smokers (28%; n = 25), with a statistically significant difference between the two groups (P = .048). Those tumor patients who displayed hypermethylation of the $p16^{INK4a}$ promoter comprised 13 with squamous cell carcinoma (59%; n = 22), three with adenocarcinoma (60%; n = 5), one with large-cell carcinoma (50%; n = 2), one with undifferentiated NSCLC (50%; n = 2), and eight with SCLC (40%; n = 20) (Table 1).

The size of the tumor at the time of bronchoscopy had a clear effect on our ability to detect *p53* and K-*ras* mutations (Table 2). Of 15 patients with limited disease (stages I to IIIA), only one K-*ras* mutation (in a patient with stage II disease) and one *p53* lesion (in a patient with stage IIIA disease) were found. In these two cases, both tumors were larger than 3 cm. In contrast, epigenetic modification of $p16^{INK4a}$ was detected in 11 samples (73%; n = 15) from patients with tumors at or below tumor stage IIIA, as well as in 15 (42%) of 36 patients with stage IIIB or IV disease.

Table 3 shows a comparison of the number of lesions found in each of the types of exfoliative material analyzed. The frequency of genetic lesions detected in sputum samples was comparable to that detected in either BAL or brushings taken during bronchoscopy. This applied to all three genetic lesions analyzed in patients with lung cancer as well as chronic smokers. Notably, the frequency of $p16^{INK4a}$ promoter hypermethylation detected in both patients with lung cancer and chronic smokers was highest in sputum.

Table 3. Frequency of Genetic Lesions Detectable in Different Types of Exfoliative Material for the Chronic Smoker and Patients With Lung Cancer Groups

	Patients With Tumors (n = 51)		Chronic Smokers (n = 25)	
	No.	%	No.	%
Sputum				
K-*ras* mutation	5	10	0	
p53 mutation	4	8	1	4
$p16^{INK4a}$ hypermethylation	18	35	4	16
BAL				
K-*ras* mutation	6	12	0	
p53 mutation	4	8	2	8
$p16^{INK4a}$ hypermethylation	11	22	3	12
Brushings				
K-*ras* mutation	4	8	0	
p53 mutation	5	10	0	
$p16^{INK4a}$ hypermethylation	8	16	2	8

To investigate whether the three genetic lesions occurred in combination in either of our patient groups, we analyzed the total number of events found in each patient (Table 4). One of the tumor patients, but none of the chronic smokers, bore all three genetic alterations. Two of the investigated genetic lesions were detected simultaneously in four tumor patients (8%) and two chronic smokers (8%). A single genetic lesion was detected in 30 tumor patients (59%) and six chronic smokers (24%).

The redundancy of genetic alterations throughout the different levels of material examined was analyzed to gain information about the expansion and dissemination of clonal cells that harbored the lesions. As summarized in Table 5, a given genetic lesion in the chronic smoker group was found exclusively at one level of the exfoliative material examined (sputum, BAL or brushings), with one exception, in which $p16^{INK4a}$ hypermethylation was detected in sputum and BAL. Clinical follow-up of this patient revealed the development of lung cancer after bronchoscopy. The same genetic lesion was detected at more than one level (sputum, BAL or brushings) in 17 tumor patients, of whom five demonstrated the alteration at all three levels.

Table 4. Synopsis of Genetic Lesions (K-*ras* and *p53* Mutations, $p16^{INK4a}$ Hypermethylation) Detected in Exfoliative Material From Patients With Tumors and Chronic Smokers

	No. of Mutations					
	3		2		1	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Patients with tumors (n = 51)	1	2	4	8	30	59
Chronic smokers (n = 25)	0		2	8	6	24

To establish whether the genetic lesions identified within the exfoliative material could be attributed to the tumor itself, DNA was extracted from paraffin-embedded biopsies from 21 patients with tumors and three chronic smokers (numbers were limited by the unavailability of biopsies or inadequate DNA quality) and analyzed for $p16^{INK4a}$ promoter hypermethylation and K-*ras* and *p53* mutations. Hypermethylation of the $p16^{INK4a}$ promoter could be confirmed within the tumor biopsy in four of the five cases in which it was detected within exfoliative material. K-*ras* and *p53* mutations were found in three of four and two of six tumors, respectively, for which corresponding exfoliative material gave a positive result. No genetic alterations were detected within any of the three biopsies obtained from chronic smokers, although *p53* mutation had been identified within exfoliative material from two of these cases. In no case did analysis of the biopsies of the tumor patients or chronic smokers reveal any genetic alteration that had not been identified within exfoliative material.

DISCUSSION

In this study, we compared the frequency of three genetic alterations (*p53* and K-*ras* mutations and $p16^{INK4a}$ promoter hypermethylation) in different types of exfoliative material obtained from chronic smokers and patients with lung cancer. Our results demonstrate a significant frequency of lesions (32% overall; Table 1) in chronic smokers of greater than 20 pack-years, who are considered to be at high risk of development of lung cancer. The absolute number of genetic alterations detectable, however, was significantly higher in patients with lung cancer than in chronic smokers (69% *v* 32% for any lesion). This finding is in line with the concept of field cancerization. Redundancy of events, whereby the same genetic lesion is detected at multiple sampling levels, was a feature of the lung cancer group, presumably because of clonal expansion.

Hypermethylation of the $p16^{INK4a}$ promoter was the most frequently detected alteration of the three markers investigated in exfoliative material, both in patients with lung cancer and in chronic smokers. The presence of this epigenetic lesion was also confirmed within the tumor material itself in the majority of cases examined, which indicates that $p16^{INK4a}$ promoter hypermethylation is not an incidental finding consequent to generalized epithelial damage, but rather results from clonal expansion of tumor cells. The frequency of $p16^{INK4a}$ promoter hypermethylation was higher in patients with confirmed lung cancer than in chronic smokers (35% and 16%, respectively, assayed in sputum), which suggests that it is not a prerequisite for neoplastic transformation in the lung but that, when it occurs, it does so as an early event. This is supported by the

Table 5. Frequency With Which a Genetic Lesion (K-*ras* and *p53* mutations, or $p16^{INK4a}$ Hypermethylation) Was Detected at One, Two, or Three Levels of Sampling (Sputum, BAL, Brushings) in Chronic Smokers or Patients With Lung Cancer

	Levels of Detection					
	3		2		1	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Patients with tumors (n = 51)						
K-*ras* mutation	1	2	5	10	2	4
p53 mutation	2	4	1	2	4	8
$p16^{INK4a}$ hypermethylation	2	4	6	12	18	35
Chronic smokers (n = 25)						
K-*ras* mutation	0		0		0	
p53 mutation	0		0		3	12
$p16^{INK4a}$ hypermethylation	0		1	4	6	24

finding that a high proportion of patients with early-stage (stages I to IIIA) lung cancer exhibited $p16^{INK4a}$ promoter hypermethylation (Table 2), with frequencies similar to those found by Ahrendt et al[31] in their analysis of bronchial lavage from patients with early-stage lung cancer. Our findings also corroborate and expand on the results from a recent study by Belinsky et al,[32] which investigated $p16^{INK4a}$ hypermethylation in sputum from a group of chronic smokers and seven patients with lung cancer and showed for the first time the presence of *p16* hypermethylation in individuals at risk of lung cancer. Our observations from a larger collective provide evidence that aberrant $p16^{INK4a}$ promoter hypermethylation accumulates during preneoplasia and early stages of lung cancer. Thus, it may constitute a candidate marker to enable detection of early lung cancer or even risk of disease in chronic smokers.

The presence of the investigated *p53* hot spot mutations did not differentiate between patients with tumors and chronic smokers (P = .572). Both these groups demonstrated a similar frequency of *p53* lesions (14% and 12%, respectively). In agreement with other investigators,[42,43] we did not find the presence of *p53* mutation to be correlated with any histologic subgroup. Interestingly, we could not confirm the presence of *p53* mutation detected in exfoliative material within corresponding tumor biopsy material in two thirds of cases investigated. This raises doubts that such a genetic alteration is attributable to the tumor itself. Instead, it is possible that *p53* mutation arises from incidental generalized epithelial damage. Such genetic alterations have been shown to occur within the bronchial epithelium of cancer-free chronic smokers and to be unrelated to any malignancy.[44] Thus, the specificity of *p53* mutation as a candidate marker of lung cancer is not optimal. Furthermore, despite analyzing three hot spot mutations in this study, only 20% to 25% of the *p53* mutations which are recognized to play a role in lung cancer can be expected to be detected with this protocol. This highlights the challenges of incorporating *p53* mutation analysis into a screening program for lung cancer.

The distribution of K-*ras* lesions differed from that of *p53* mutations and $p16^{INK4a}$ promoter hypermethylation. K-*ras* mutations in codon 12 were found exclusively in the tumor group and were restricted to NSCLC histologies (Table 1). This supports the findings of several other groups who have reported the association of K-*ras* mutation with lung cancer.[26,45] Our findings do not agree with those reports that demonstrate K-*ras* lesions in noncarcinogenic tissue of the lung.[46-49] This apparent discrepancy might arise from the use of differing types of patient material. Our investigation was performed on exfoliative material, and therefore, contamination with nontumor material might have influenced the sensitivity of our assays. Furthermore, although all studies applied DNA amplification protocols, the level of sensitivity is not necessarily comparable. For example, extremely sensitive enriched PCR protocols were applied in some studies, which allowed the detection of less than 1 allele in 1,000 normal alleles.[47,49] Perhaps these highly sensitive conditions account for the detection of K-*ras* mutations in nononcologic patients.[47,49] The relatively low frequency of K-*ras* mutation in lung cancer, however, precludes its use as a marker in isolation.

Although the sensitivity for any of the markers of lung cancer investigated in this study was relatively low, with frequencies ranging between 14% and 51% for the tumor group (Table 1), combining the three markers increased the detection rate to almost 70%. In line with our results, the use of four independent molecular markers was shown to increase the sensitivity, enabling detection of more than 90% of lung cancers (J. Herman, personal communication, April 1999). There is increasing evidence that clonal evolution of tumors from premalignant lesions is a complex process that involves multiple genetic abnormalities, which do not necessarily have a linear progression.[50] Thus, as our results intimate, effective detection of preneoplasia or early lung cancer is likely to mandate the use of a panel of molecular markers, spanning the various genetic alterations that might be present, rather than being feasible with single markers alone.

Analysis of tumor DNA did not reveal any lesion that had not been detected in exfoliative material. A further aim of this study was to evaluate the suitability of DNA obtained from sputum compared with that from bronchial lavage fluid or brushings for monitoring genetic and epigenetic alterations in the bronchial tree. Our results show that analysis of sputum, obtained by noninvasive means, offers a

comparable level of detection as samples taken by invasive bronchoscopy. In fact, a higher frequency of $p16^{INK4a}$ promoter hypermethylation was detected for chronic smokers, as well as patients with lung cancer, in sputum than in either bronchial lavage or brushings, which perhaps reflects the merits of screening a sample that is independent of anatomic location within the bronchial tree. Therefore, sputum could provide a valuable source of material for routine screening for lung cancer. Recently, abnormal DNA methylation was found in the serum of patients with lung cancer at all tumor stages at relatively high frequencies.[51] Because release of tumor-derived DNA into the circulation requires efficient tumor vascularization, however, screening of serum is unlikely to be able to detect preinvasive lesions early enough to improve the effects of therapeutic intervention. Thus, for the monitoring of individuals at high risk of lung cancer, sputum seems to be the best candidate material.

Finally, it is important to establish whether the genetic alterations investigated constitute markers for a risk of developing lung cancer or for established malignancy. We have approached this issue in two ways. As discussed above, DNA from tumor biopsies for approximately one half of our study collective was analyzed to investigate whether the genetic lesions detected in exfoliative material could be confirmed in and, thus, attributed to the tumor. This analysis demonstrated that the majority of K-*ras* and $p16^{INK4a}$ lesions found in exfoliative material of patients with lung cancer matched those found in primary tumor material. Because no such biopsy material was available for the chronic smokers, we observed their clinical history. Of eight patients with genetic lesions at the time of bronchoscopy, three cases subsequently developed a de novo cancer. Patient no. 028 developed lung cancer 1 year after bronchoscopy and is now deceased. Patient no. 052 developed cancer of the esophagus occluding the left main bronchus 14 months after bronchoscopy. Patient no. 078, who had a history of larynx carcinoma resected 12 years before, developed metastasizing lung cancer 1 year after bronchoscopy, of which he died. All three of these patients exhibited $p16^{INK4a}$ promoter hypermethylation at the time of bronchoscopy. In addition, patient no. 028 bore a *p53* mutation in codon 273. Although this evidence is circumstantial, it gives hope that detection of $p16^{INK4a}$ promoter hypermethylation in particular may be of value to the clinician in managing symptomatic chronic smokers. Such patients may warrant more regular follow-up with conventional diagnostic investigations. Clearly, prospective clinical studies are required to evaluate the potential benefit of such a marker in accelerating the diagnosis of lung cancer and thus maximizing the chances of effective therapeutic intervention.

In summary, this is the first study to compare detection of $p16^{INK4a}$ promoter hypermethylation and *p53* and K-*ras* mutations in collectives of symptomatic chronic smokers and patients with different stages of lung cancer and to evaluate the use of exfoliative material for such analyses. K-*ras* mutation was found exclusively in the lung cancer group and is thus a candidate marker for the presence of neoplastic transformation. Although *p53* mutation was detected in exfoliative material from both patients with lung cancer and symptomatic chronic smokers, its significance is unclear because this change was not necessarily attributable to the tumor material. We have shown that $p16^{INK4a}$ promoter hypermethylation occurs in chronic smokers in the absence of clinical evidence of neoplasia and that its frequency increases further in early stages of lung cancer. Therefore, hypermethylation of the $p16^{INK4a}$ promoter may constitute a marker of increased risk of developing lung cancer or of early disease.

This study has shown that analysis of sputum yields information equivalent to that obtained from bronchial lavage or brushings. Therefore, molecular analysis of sputum for a panel of markers including $p16^{INK4a}$ promoter hypermethylation and K-*ras* mutation may provide an effective means of screening symptomatic chronic smokers to enable earlier detection and therapeutic intervention of lung cancer.

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APPENDIX G

Diagnosis of Renal Cancer by Molecular Urinalysis

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Background: Organ-confined renal malignancies can be cured in the majority of patients, whereas more extensive lesions have a poor prognosis. We sought to develop a noninvasive test for renal cancer detection based on a novel molecular approach. _Methods:_ Matched urine and serum DNA samples were obtained before surgery from 30 patients with clinically organ-confined solid renal masses (25 with malignant tumors and five with tumors of low malignant potential) and were subjected to microsatellite analysis. Serum samples and urine samples obtained from 16 individuals without clinical evidence of genitourinary malignancy served as controls. _Results:_ Nineteen (76%) of the 25 patients with malignant tumors were found to have one or more microsatellite DNA alterations in their urine specimen, and 15 (60%) were found to have alterations in their serum DNA by microsatellite analysis. In every case, the microsatellite changes in urine or serum were identical to those found in the primary tumor. Three of five patients with tumors of low malignant potential were found to have DNA alterations in their urine, but none displayed alterations in their serum. Moreover, microsatellite alterations were not identified in either the urine or the serum samples from normal control subjects and patients with hematuria due to nephrolithiasis (renal stones). _Conclusion:_ These data suggest that microsatellite DNA analysis of urine specimens provides a potentially valuable tool for the early detection of resectable kidney cancer. Furthermore, microsatellite analysis of serum samples reveals evidence of circulating tumor-specific DNA in approximately half of these patients and may reflect the propensity of these tumors to spread to distant sites at an early stage. [J Natl Cancer Inst 1999; 91:2028–32]

Approximately 30 000 new cases of malignant renal cancer are diagnosed each year in the United States, and nearly 12 000 individuals succumb to the effects of metastatic disease annually *(1)*. Although organ-confined renal carcinoma can be cured by radical nephrectomy, carcinoma that extends beyond the confines of the renal capsule is associated with substantial morbidity and a high rate of cancer-specific mortality *(2)*. The application of conventional adjuvant therapies to patients with advanced disease has been disappointing because of the resistance of most malignant renal tumors to standard chemotherapy and radiotherapy *(2)*.

The specific clinical signs and symptoms of malignant renal disease are not usually helpful in making an early diagnosis. The classic triad of pain, hematuria, and a palpable flank mass is encountered in only 10% of patients and is usually associated with the presence of advanced disease *(3)*. The widespread use of noninvasive axial imaging (e.g., computed tomography or magnetic resonance imaging) and diagnostic ultrasound has resulted in the incidental discovery of an increasing number of small asymptomatic renal tumors. Estimates suggest that as many as two thirds of organ-confined tumors are identified by serendipity *(4)*. Nevertheless, 50% of patients with renal cancers are not curable by surgical resection at the time of presentation. Consequently, the development of a reliable, noninvasive method for the early detec-

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See "Notes" following "References."

ın of kidney cancer could represent an ıportant clinical advance in the management of this patient population.

Microsatellite analysis is a polymerase ain reaction (PCR)-based technique at permits the detection of cancerecific DNA alterations (loss of heterogosity [LOH] and microsatellite instality) in neoplastic tissue. Recent ıplication of this approach to the evaluion of body fluids has shown that squaous cell carcinoma of the aerodigestive ıct and bladder cancer can be detected rough the analysis of saliva and urine, spectively *(5–7)*. Since the renal parenıyma is highly vascular and lies in close ıysical proximity to the renal collecting stem and since the tubular epithelium om which most malignant renal neoasms arise contributes directly to urine rmation, we hypothesized that DNA alrations characteristic of malignancy ould be identified by microsatellite ıalysis of either serum or voided urine ecimens obtained from patients with real malignancies. To test this hypothesis, e studied, by use of microsatellite analys, preoperative urine and serum speciens obtained from patients with a variy of renal neoplasms.

IATERIALS AND METHODS

Sample collection and DNA isolation. After we ıtained written informed consent from 30 patients ith a renal lesion, samples of peripheral blood and ine were collected before surgical intervention. ıe study was approved by the Institutional Review ɔard of The Johns Hopkins Hospital. Neoplastic dney tissue was obtained immediately after surgil resection and stored at −80 °C. Eight control mples were obtained from patients with nephroliiasis (renal stones) and eight from individuals ithout a history of genitourinary disease (total =). Tumor tissue was microdissected as previously scribed *(8)*. DNA was obtained from peripheral mphocytes, serum, and tumor samples by digeson with the use of proteinase K (Boehringer Mannim GmbH, Mannheim, Germany) in the presence sodium dodecyl sulfate at 48 °C overnight, folwed by phenol–chloroform extraction and ethanol ecipitation.

Microsatellite analysis. Based on high rates of formativity and known patterns of LOH and miosatellite instability in renal cancer, 28 microsatlite markers (Research Genetics, Huntsville, AL) ere identified for use in this series of experiments. licrosatellite markers (and their chromosomal loıtions) are as follows: D1S251 (1pq), HTPO (2p), 3S1317 (3p), D3S587 (3p), D3S1560 (3p), 3S1289 (3p), D3S1286 (3p), D3S1038 (3p), 4S243 (4pq), FGA(4) (4q), CSF (5q). ACTBP2 p), D8S348 (8q), D8S307 (8p), D9S747 (9p), 9S242 (9p), IFN a (9p), D9S162 (9p), D11S488 1q), THO (11p), vWA (12p). D13S802 (13q), JD (14q), D17S695 (17p). D17S654 (17p), D18S51 (18q), MBP (18q), and D21S1245 (21q). Primer sequences and locations were obtained from the Genome Database (The Johns Hopkins University, Baltimore, MD). One primer from each pair was end-labeled with [γ-^{32}P]adenosine triphosphate (Amersham Life Science Inc., Arlington Heights, IL) with the use of bacteriophage T4-polynucleotide kinase (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). Genomic DNA (50 ng) was subjected to 35 PCR cycles at a denaturing temperature of 95 °C for 1 minute, followed by varying annealing temperatures (52 °C–60 °C, depending on the primer sequence) for 1 minute, an extension step at 72 °C for 1 minute, and a final extension step at 72 °C for 5 minutes by use of a thermocycler (Hybaid, Teddington, U.K.). PCR products were then separated in denaturing 7% polyacrylamide–urea–formamide gels, followed by autoradiography for 12–36 hours on X-omat film (Eastman Kodak Co., Rochester, NY) *(9)*. LOH was determined by a comparison of the intensity of the allelic bands from nonmalignant (lymphocyte) DNA with that of the allelic bands from the target sample (from tumor, urine, or serum). A reduction in the intensity of one allele in the target sample of more than 50% (30% in serum as a result of a greater dilution with normal DNA), as assessed by two independent observers (C. F. Eisenberger and D. Sidransky), was considered to represent LOH and the presence of new "shifted" alleles (appearance of new bands) as microsatellite instability. Clinical data were obtained from the patient charts. Every marker alteration (LOH or microsatellite instability) was confirmed by reamplification of the starting material and independent verification by two separate observers.

Statistical analysis. The sensitivity and specificity of marker alterations in urine and serum were calculated as follows: sensitivity = number of positive tests/number of cancer cases, and specificity = number of negative tests/number of cases without cancer. The proportion of patients showing a microsatellite alteration was compared between tumor stages with the use of Fisher's exact test. *P* values reported are two-sided.

RESULTS

We tested 28 microsatellite markers from 20 chromosomal regions in paired urine and serum specimens from case patients with cancer and control subjects. Nineteen (76%) of 25 patients with malignant tumors of the kidney (Table 1) were found to have one or more microsatellite alterations (microsatellite instability and LOH combined) in the urine DNA, and 15 (60%) of these 25 patients were found to have at least one alteration (microsatellite instability and LOH combined) in the serum DNA (Fig. 1, Table 1). In each case, the identical genetic alteration was also found in the corresponding DNA from the tumor sample. Three (60%) of the five patients with tumors of low or negligible malignant potential (one angiomyolipoma and two oncocytomas) displayed LOH at one or more markers in the urine that also matched the pattern of LOH found in the corresponding tumor (Table 1). None of the eight healthy subjects and none of the eight patients with nephrolithiasis displayed alterations in the urine or serum. Thus, detection of microsatellite alterations in the urine had a sensitivity of 73% (22 of 30 patients in this study) for identifying patients with a renal mass and 100% specificity. The corresponding sensitivity and specificity for serum were 50% (15 of 30) and 100%, respectively.

One patient with transitional cell carcinoma (TCC) of the renal pelvis was included in the present analysis (#1290, Table 1). DNA changes were readily detectable in the urine and serum samples obtained from this patient preoperatively. The finding of readily identifiable LOH in the urine of this patient is consistent with previously published results obtained from studies of microsatellite analysis in patients with TCC of the bladder *(6,7)*. Unlike patients with bladder cancer (Linn CJ, Sidransky D: unpublished data), however, this patient with cancer of the renal pelvis displayed serum abnormalities as well (Table 1).

There was no association between evidence of either microsatellite instability or LOH in serum and the stage of tumor (Table 2). Fifteen (60%) of 25 patients treated for malignant renal neoplasms in this study had evidence of either LOH or microsatellite instability in serum samples obtained preoperatively. Of these 15 patients, three had tumors of stage T1, eight had tumors of stage T2, and four had tumors of stage T3, and there was no difference in serum detection between any of these tumor stages (*P* = .39, Fisher's exact test). Similarly, the urine samples were positive in 19 patients, including four patients with stage T1 tumor, 11 with stage T2 tumor, and four with stage T3 tumor. Again, there was no association between stage of tumor and evidence of microsatellite alterations in urine specimens (*P* = .39, Fisher's exact test). Patients with lesions of low malignant potential failed to show evidence of serum alterations in this analysis.

DISCUSSION

Pathologic stage of disease predicts an individual patient's clinical outcome more profoundly than any other currently available marker after surgical treatment for a malignant renal tumor. Radical nephrectomy remains the primary mode of therapy for kidney cancers, although le-

Table 1. Samples showing loss of heterozygosity (LOH) and/or microsatellite instability in the tumor, urine, and serum

).	Pathology*	Age, y	pTNM†	Grade‡	Symptoms/history§	LOH, tumor/urine/serum‖	Microsatellite instability, tumor/urine/serum‖
30	RCC, clear cell	51	T2N0MX	II–III	Hematuria	6/0/0	2/0/0
10	RCC, chromophobe	52	T2N0MX	II–III	Microscopic hematuria	6/1/0	1/0/0
45	RCC, clear cell	65	T3bN0M1	III	Metastasis (lung, subcutaneous)	2/0/0	0/0/0
53	RCC, clear cell	72	T3aNXMX	III	None	2/1/1	0/0/0
83	RCC, clear cell	65	T3aNXMX	II	Discomfort	4/1/1	0/0/0
70	RCC, clear cell	74	T2NXMX	II	Glomerulosclerosis	5/1/1	1/1/1
17	RCC, clear cell	58	T2NXMX	II	Pain, CIS of glans, cholelithiasis	13/8/1	0/0/0
50	RCC, clear cell	33	T2NXMX	I	Hematuria/pain	4/0/0	1/1/0
61	RCC, clear cell	70	T1NXMX	I–II	None	4/2/1	1/1/1
63	RCC, clear cell	61	T2NXMX	II	Hematuria	5/2/0	0/0/0
94	RCC, clear cell	75	T2NXMX	II	Recurrent UTI, hematuria	7/4/1	0/0/0
99	RCC, clear cell	67	T3bN1MX	II–III	Discomfort/mass	4/3/1	0/0/0
23	RCC, clear cell	60	T2N0MX	II	None	6/1/1	0/0/0
24	RCC, clear cell	46	T2NXMX	II–III	None	1/0/0	0/0/0
40	RCC, clear cell	45	T2NXMX	I	None	2/1/1	1/1/1
60	RCC, clear cell	59	T2NXMX	I	None	2/0/1	0/0/0
75	RCC, clear cell	60	T1N0MX	I–II	None	2/2/0	1/0/0
49	RCC, clear cell	61	T2N0MX	II	None, renal pelvis involved	4/0/0	0/0/0
13	RCC, clear cell	74	T2N0MX	II	None	3/1/1	0/0/0
34	RCC, clear cell	67	T1N0MX	I	None	3/2/1	0/0/0
52	RCC, hypernephroid	69	T3NXMX	III	None	5/3/2	0/0/0
290	TCC, renal pelvis	76	T1NXMX	III	Hematuria/positive cytologic specimen	13/8/3	1/1/0
152	RCC, papillary	70	T2NXMX	III	None, collecting ducts involved	20/3/0	0/0/0
51	RCC, papillary	63	T2N0MX	II	None	4/0/0	0/0/0
50	RCC, papillary	54	T2N0MX	II	None	11/3/2	0/0/0
500	Angiomyolipoma	63	4 cm		None	5/2/0	1/0/0
522	Metanephric nephroma	68	4 cm		None	1/0/0	1/0/0
158	Oncocytoma	77	2 cm		Hematuria/pain	1/1/0	0/0/0
540	Oncocytoma	74	2.7 cm		None	1/1/0	0/0/0
514	Oncocytoma	63	4 cm		None	1/0/0	0/0/0

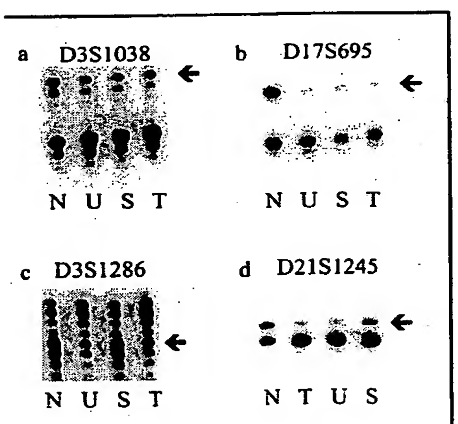
*RCC = renal cell carcinoma; TCC = transitional cell carcinoma.

†American Joint Committee on Cancer staging. pTNM: p = pathologic stage; T = tumor size; N = node status; M = metastatic status.

‡American Joint Committee on Cancer.

§CIS = carcinoma *in situ*; UTI = urinary tract infection.

‖Numbers under LOH and microsatellite instability columns indicate positive microsatellite markers in tumor, urine, and serum DNA. In total, 28 markers were sted by microsatellite analysis in each clinical sample. All samples are from patients with malignant tumors, except those from patients 1500, 1522, 1458, 1640, ıd 1614 whose tumors have low or negligible malignant potential.



'ig. 1. Microsatellite analysis of clinical samples :om patients with renal cancer. Loss of the upper lleles indicating loss of heterozygosity in the urine U), serum (S), and tumor (T) in patients 1523 (a), 290 (b), and 1540 (d). Loss of the bottom allele is hown only in urine and tumor but not in serum in atient 1499 (c). Microsatellite markers are depicted ver each panel. N = normal lymphocyte DNA.

Table 2. Microsatellite analysis of clinical samples by stage of tumor at time of surgery

Sample	Stage of tumor* T1 (n = 4)	T2 (n = 16)	T3 (n = 5)	P†
Urine	4 (100)	11 (69)	4 (80)	.39
Serum	3 (75)	8 (50)	4 (80)	.39

*Number (%) with loss of heterozygosity or microsatellite instability in sample.

†Two-sided, Fisher's exact test.

sions extending beyond the kidney are substantially more difficult to cure than are those limited to the confines of the renal capsule. Although noninvasive imaging has clearly improved clinical staging of renal tumors, neither ultrasound nor computed tomography is sufficiently inexpensive or accessible to be used for widespread early-detection programs.

Advances in basic research have shed light on some events that putatively contribute to the development of renal neoplasia. Detailed studies of pathology *(10)* have underscored the morphologic heterogeneity of renal cancers. Genetic studies employing a variety of technologies *(11–13)* have shown that renal cancers are characterized by specific chromosomal abnormalities, the most common of which include subchromosomal losses on 3p in sporadic clear cell carcinomas, aneuploidy of chromosomes 7 and 17 in papillary renal tumors, and small deletions on chromosomal arm 1p in oncocytomas and on distal 1q in collecting duct carcinomas. Elegant familial studies of von Hippel–Lindau (VHL) disease have led to the identification of the gene putatively responsible for that disease located at 3p25 *(14)*. Additional work *(15)* has shown that LOH at the VHL locus may be characteristic of most sporadic renal cancers. However, these advances in basic research have not yet translated into the development of reliable diagnostic markers of renal cancer.

The central clinical problem facing rgeons and oncologists who care for pants with renal cancer is that this cancer unresponsive to conventional systemic juvant therapies, unlike other genitourity cancers for which successful adjunt therapies have been developed (i.e., s-platinum-based chemotherapeutic gimens for bladder cancer and nonsemimatous testicular neoplasms) *(16,17)*. ıdioresistance is also characteristic of ıal tumors, leaving surgery as the sole, nsistently successful form of therapy. ırgical removal of the kidney has a nited role in the treatment of patients th advanced disease and is often not rative in patients with tumors that exıd beyond Gerota's fascia or that inılve regional lymphatics. Although proective trials have not yet confirmed e value of early renal cancer detection, ıge-specific survival data suggest that, renal neoplasms could be consistently tected at the organ-confined stage, the sease-specific survival rate could be pected to increase. It is interesting to ıte that a recent retrospective autopsy ries identified renal cancer as one of e most common undiagnosed tumors at contributed to death in U.S. patients ıo underwent postmortem examination *8)*.

The ease with which microsatellite alysis can be performed on a variety DNA sources continues to increase. ıe molecular diagnosis of renal canr by urinalysis is remarkable in that ecimens from all patients contained a rtual absence of cellular material or ıly scant cellular fragments. Further enhment of neoplastic cell (or cell fragent) populations in the urine with antiıdies to cancer-specific antigens such as N/CA9 protein *(19)* may further inease the sensitivity of microsatellite alysis. Evolving knowledge of the getic changes that drive kidney cancer ogression and the ability to distinguish alignant and benign tumors of the dney will also lead to further improveents *(20)*. We have demonstrated e ability to perform multiple analyses ing a new high-throughput micropillary array that can markedly enhance e potential clinical utility of this mocular diagnostic approach *(21)*. Translaın of this type of technology to the inical laboratory may hold the key to oader application and eventual use microsatellite-based cancer diagnoss.

Urologic malignancies have well-studied patterns of anatomic spread, the best characterized of which are testis and prostate cancers *(3)*. Both of these tumor types are known for their propensity to metastasize by lymphatic routes. In contrast, renal cancer probably spreads by a combination of lymphatic and hematogenous mechanisms, a fact underscored by the finding that circulating cancer cells have been readily extracted from the blood of patients with various stages of malignant renal disease *(22)*. The finding of serum microsatellite alterations in greater than 50% of the patients studied for this report probably reflects the hematogenious mechanism by which certain renal tumors spread. A recent report *(23)* confirmed a similar frequency of microsatellite alterations in the serum of patients with clear cell renal tumors. While it is not yet possible to say to what extent patterns of microsatellite alterations are related to the malignant and metastatic potential of individual renal tumors, further study of these tumors with different panels of markers may reveal patterns of LOH and microsatellite instability with prognostic as well as diagnostic potential. Coupled with high-throughput technologies, genome-wide search strategies, and the opportunity to study a larger population of patients in a multi-institutional venue, microsatellite analysis may permit the identification of patients with early and potentially resectable disease. Microsatellite alterations in serum predicted a poor prognosis in patients with head and neck cancer *(24)* and may also identify kidney cancer patients at risk for disease progression for whom experimental adjuvant therapies may be beneficial.

We have demonstrated that microsatellite analysis of urine can frequently detect the presence of malignancy in patients with clinically organ-confined renal cancer. Patients with renal lesions of lower malignant potential, such as oncocytomas, demonstrated DNA alterations in matched urine samples but not in preoperative serum samples. Individuals with nephrolithiasis and control subjects without genitourinary complaints or symptoms did not have positive microsatellite analyses in this study. Larger multicenter trials utilizing this assay for the diagnosis and potential staging of renal cancer patients are warranted to determine the ultimate clinical utility of this molecular approach.

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APPENDIX H

Disseminated Tumor Cells in Pancreatic Cancer Patients Detected by Immunocytology: A New Prognostic Factor[1]

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ABSTRACT

Using an immunocytological approach, we previously showed that disseminated cancer cells are frequently found in peritoneal cavity and bone marrow samples of gastrointestinal and pancreatic cancer patients. Recently, we demonstrated that the detection of isolated tumor cells could serve as a new prognostic factor in gastric and colorectal cancer. Thus far, no conclusive data concerning the clinical implication of minimal residual disease in pancreatic cancer exist. In this study, we investigated peritoneal lavage and bone marrow samples of 80 pancreatic cancer patients to determine the predictive value of immunocytologically detected disseminated tumor cells. Therefore, immunocytological findings were correlated with the clinical follow-up data (median observation time, 10.7 months; range, 2–61 months), and the findings in peritoneal cavity and bone marrow samples were compared. Fifty-two % of the patients showed minimal residual disease at least in one compartment (39% positive lavage and 38% positive bone marrow samples). The detection rate of isolated tumor cells increased in parallel to the tumor stage. The presence of tumor cells in the peritoneal cavity significantly correlated with the survival time of the patients ($P = 0.0035$). In bone marrow samples, a strong trend was seen ($P = 0.06$). The evaluation of both compartments increased the number of positive patients and resulted in a highly significant correlation: all patients who were positive in at least one compartment died within 18 months, whereas negative patients showed a 5-year survival rate of 30% ($P < 0.0001$). We recommend immunocytological investigation of peritoneal cavity and bone marrow samples as a new prognostic marker in pancreatic cancer patients.

INTRODUCTION

The prognosis of pancreatic cancer is extremely poor; the overall 5-year survival rate is only 2%. A chance of cure exists only for a minority of patients with locally limited and surgically resectable tumors (1). However, of patients who are radically treated by a partial pancreatico-duodenectomy and lymphadenectomy (R0 resection), 70–80% will suffer from an incurable local relapse, distant metastases, or peritoneal carcinosis (2). Although a local relapse might be caused by an incomplete resection, distant metastases and peritoneal carcinosis ultimately depend on a dissemination of malignant cells. Presently, those cells are not detectable with conventional diagnostic tools. However, their elimination is the aim of various adjuvant therapy concepts that are currently under investigation, including chemotherapy (3), immunotherapy (4), and gene therapy (5). For a specific selection of patients who would benefit from those therapies, it would be helpful to detect minimal residual disease.

Immunocytological methods, which are significantly more sensitive than conventional cytology (6), have made it possible to detect disseminated tumor cells in the bone marrow of patients with breast cancer (7), small cell lung cancer (8), neuroblastoma (9), prostatic cancer (10), gastric cancer (11), and colorectal cancer (12). Previously, we could demonstrate that minimal residual disease also frequently occurs in bone marrow of pancreatic cancer patients (13, 14). However, bone marrow metastases are a rare event in pancreatic cancer (15). Disseminated tumor cells are more likely to occur in the peritoneal cavity because of the high frequency of local relapse or peritoneal carcinosis. Therefore, we also developed an immunocytological approach to detect isolated tumor cells in peritoneal cavity samples (13). Recently, we showed that the finding of tumor cells in the peritoneal cavity can serve as a prognostic marker in gastric and colorectal cancer (16). However, no comprehensive immunocytological studies exist concerning the clinical implication of isolated i.p. and bone marrow tumor cells in pancreatic cancer.

This study was initiated to determine the predictive value of disseminated tumor cells in pancreatic cancer patients. We extended our former studies to a larger collection of patients and investigated peritoneal lavage and, for comparison, bone marrow samples in surgically treated tumor patients and compared the immunocytological findings with the clinical data.

Here, we show that tumor cells were frequently detectable in the peritoneal cavity and in the bone marrow. A finding of isolated tumor cells correlated significantly with the postoperative survival rate of pancreatic cancer patients.

MATERIALS AND METHODS

Patients

All patients were extensively informed and gave written consent for the investigations, including the bone marrow aspi-

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ration. The study was confirmed by the ethics commission of the University Hospital Kiel (Kiel, Germany).

We evaluated the data of 80 patients who suffered from an adenocarcinoma of the pancreas and underwent surgery (carcinoma of the papilla vateri and endocrine tumors were excluded). Bone marrow samples were tested in 71 patients (9 patients declined to give their consent), and lavage samples could be obtained in 62 patients (18 patients showed adhesions that were too extensive). Pairs of peritoneal lavage and bone marrow were analyzed in 53 patients.

Patients who died in the hospital due to surgical complications or tumor progress ($n = 7$) and patients who attended a clinical study for adjuvant therapy ($n = 2$) were excluded from this study. Another six patients who were initially investigated were lost in the follow-up and, therefore, not included in this study (1 stage II and positive in peritoneal lavage; 2 stage III and immunocytologically negative; and 3 stage IV, 2 of which were immunocytologically positive).

Control Group. Fifty-eight patients who underwent surgery but did not suffer from a malignant disease served as a control group; 45 agreed to a bone marrow aspiration and 43 agreed to a peritoneal lavage. The control group included patients suffering from benign liver tumors ($n = 10$), sigma diverticulitis ($n = 8$), chronic pancreatitis ($n = 7$), cholecystolithiasis ($n = 5$), achalasia ($n = 4$), other benign diseases ($n = 3$), and hypersplenism ($n = 4$). Additionally, ascites from patients with liver cirrhosis ($n = 5$) and bone marrow samples from patients with benign hematological disease ($n = 12$) were examined.

Samples

Bone marrow (10 ml) was aspirated from the right spina iliaca anterior at the beginning of the operation (Jamshidi needle). Peritoneal lavage was performed before manipulation of the tumor. One liter of isotonic sodium chloride solution was instilled and immediately removed (13).

Preparation of Samples. The lavage solution was centrifuged (270 × *g* for 10 min), and the supernatant was discharged. The cell pellet was resuspended in 20 ml of RPMI 1640. All lavage suspensions and bone marrow aspirates were further processed by Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged onto microscopic slides (2.5×10^5 cells/slide). Cytospins were fixed in acetone and stored at −20°C (13).

Immunocytochemistry

Staining of cytospins was performed by the immunoperoxidase method with six different monoclonal antibodies as described previously (13): (*a*) C1P83, gold 3 epitope of CEA[3] (17); (*b*) CA19-9 (DAKO, Carpinteria, CA), determinants of Lewis blood group antigens (18); (*c*) 17-1-A (Centocor, Leiden, the Netherlands), tumor-associated membrane antigen (19); (*d*) Ra96, tumor-associated mucin antigen (20); (*e*) C54-0, epithelial membrane antigen (21): because of a cross-reactivity with a subpopulation of lymphopoietic cells in the bone marrow, C54-0 was only evaluated for peritoneal cavity samples; and (*f*) KL-1 (Immunotech, Hamburg, Germany), cytokeratins (only used in bone marrow samples). The hybridoma cells for the antibodies C1P83, Ra96, and C54-0 were produced by our laboratory, and antibodies were purified according to standard procedures (21). A total of 1.25×10^6 peritoneal lavage and bone marrow cells each were tested for every patient. The microscopic evaluation was carried out independently by two investigators. To ensure that the investigators were not influenced by the clinical data, the slides were blinded by numbers, and the investigators were, therefore, not aware of the clinical background of the samples (*e.g.*, tumor stage, control sample, and so on).

Evaluation of Data

Samples were evaluated as tumor cell positive if at least one cell reacted with one of the monoclonal antibodies. The detection rate was correlated with the Union International Contre Cancer classification of the tumor stage and the R-classification (22).

Postoperatively, patients were examined either in our outpatient clinic or by their general practitioner. Every 3 months, a clinical examination and blood tests, including tumor markers CEA and CA19-9, were performed, and every 6 months, a sonography or computed tomography scan was performed.

The projected survival rates were determined by Kaplan-Meier calculation, and calculations of significance were determined by the log-rank test.

RESULTS

Control Group. In 43 of 45 patients, no cell staining was seen in bone marrow samples with the antibodies KL-1, C1P83, Ra96, CA19-9, or 17-1-A. In two patients, single cells were stained with the antibodies KL-1, C1P83, and 17-1-A. Additionally, one patient had positive cells for CA19-9. Both patients were strongly suspected to suffer from pancreatic cancer and were, therefore, treated by a partial duodenopancreatectomy (Whipple operation). The histological analysis could not confirm this diagnosis and found a chronic pancreatitis.

The peritoneal lavage samples of patients with no malignant diseases showed, in 40 of 43 cases, no positive reaction of the applied antibodies (C1P83, Ra96, CA19-9, 17-1-A, and C54-0). Two of the positive samples were derived from the same patients mentioned above who suffered from a chronic pancreatitis and showed C54-0- and C1P8-stained i.p. cells, respectively. A third patient with liver cirrhosis and a chronic hepatitis C was positive for the antibody C54-0.

Pancreatic Cancer. Overall, minimal residual disease was detected in 42 of 80 (52%) patients; 24 of 62 (39%) had positive peritoneal cavities and 27 of 71 (38%) had positive bone marrow samples. The antibody CA19-9 showed the highest detection rate in bone marrow samples (23%), followed by C1P83 (anti-CEA) and KL-1 (13 and 12%, respectively). In peritoneal cavity samples, C1P83 (31%) and Ra96 (20%) reacted with the highest frequency. As shown previously (13), the combination of all antibodies clearly increased the detection rate in the bone marrow, peritoneal cavity, and combined evaluation (38, 39, and 52%, respectively). On average, we found 5 isolated tumor cells per peritoneal lavage sample (range, 1–95 cells) and 3 isolated tumor cells in the bone marrow (range, 1–5 cells).

[3] The abbreviation used is: CEA, carcinoembryonic antigen.

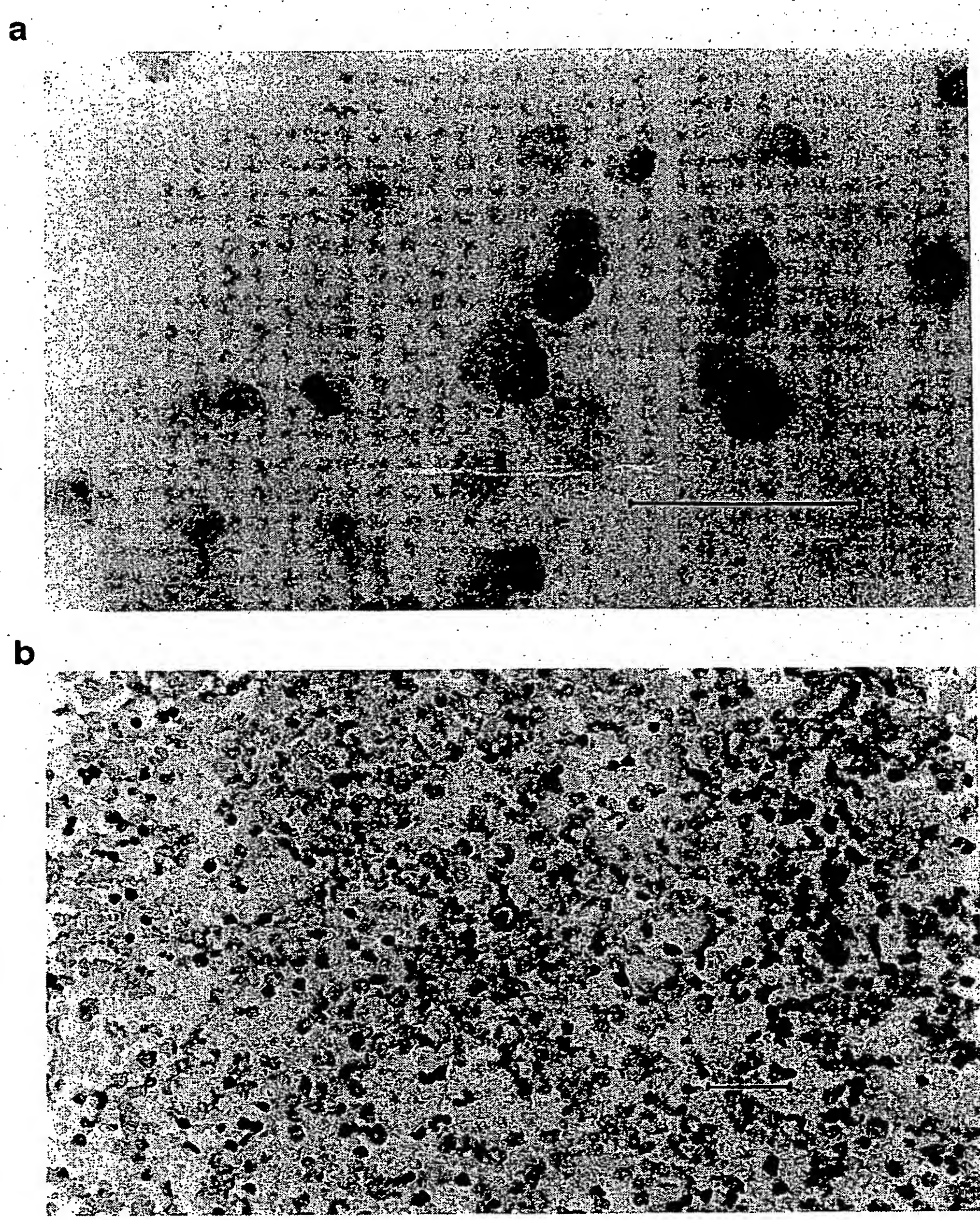

Fig. 1 Cytospins of a peritoneal lavage sample from a pancreatic cancer patient (*a*) and of a bone marrow sample (*b*) stained with CA19-9 and KL-1, respectively (*scale bar*, 50 μm).

Fig. 1 shows typical positive results in a peritoneal lavage (Fig. 1*a*) and a bone marrow sample (Fig. 1*b*). In peritoneal cavity and bone marrow samples, the detection rate increased in parallel with the tumor stage. Interestingly, one of five (20%) patients with a stage II tumor showed positive cells within the peritoneal cavity, although the tumor had no direct access to the peritoneal cavity; three of eight (38%) presented tumor cells in the bone marrow; and in three of nine (33%), the tumor had spread in both compartments.

A radical partial pancreatico-duodenectomy (Whipple procedure) including extended lymphadenectomy was performed as an R0 resection in 29 patients. In 6 patients, the histological evaluation revealed a R1 resection (microscopically remaining tumor); in 3 patients, a macroscopically incomplete tumor resection was performed (R2 resection); and 42 patients received palliative surgery by abdominal exploration and bypass operation (*e.g.*, gastroenterostomy and biliodigestive anastomosis).

In R0-resected patients, minimal residual disease was detected in 29% of the peritoneal cavity and 21% of the bone marrow samples. In incomplete resected or palliative treated patients, the detection rate was significantly higher and showed tumor cell dissemination in 56% of the peritoneal cavity and in 48% of the

Table 1 Summary of pancreatic cancer patients (n = 80) who were included in the follow-up study

Shown are the Union International Contre Cancer tumor stage; the number of patients with a "curative" operation (R0 resection), incomplete resection (R1/2 resection), or palliative operation (OP); and the detection rate in the peritoneal cavity (pc), bone marrow (bm), or either compartment (pc and/or bm).

Stage	R0 resection (n = 29)	R1/2 resection (n = 9)	Palliative OP (n = 42)	Detection rate pc	bm	pc and/or bm
I	3	0	0	0/2 (0%)	0/3 (0%)	0/3 (0%)
II	8	1	0	1/5 (20%)	3/8 (38%)	3/9 (33%)
III	13	0	6	3/13 (23%)	7/16 (44%)	9/19 (47%)
IVa	2	4	3	3/6 (50%)	3/9 (33%)	5/9 (56%)
IVb	3	4	33	17/36 (47%)	14/35 (40%)	25/40 (63%)

Table 2 Summary of 53 patients in whom peritoneal lavage and bone marrow were investigated in parallel ("pairs")

Comparison of tumor stage and number of patients (percentages in parentheses) with positive peritoneal cavity samples only (pc only), positive bone marrow samples only (bm only), patients with immunocytological findings in both compartments (pc and bm), and patients with positive findings in either compartment (pc and/or bm).

Stage	Detection rate pc only	bm only	pc and bm	pc and/or bm
I (n = 2)	0 (0%)	0 (0%)	0 (0%)	*0 (0%)*
II (n = 4)	0 (0%)	1 (25%)	1 (25%)	*2 (50%)*
III (n = 10)	1 (10%)	4 (40%)	1 (10%)	*6 (60%)*
IVa (n = 6)	2 (33%)	1 (17%)	1 (17%)	*4 (66%)*
IVb (n = 31)	9 (29%)	7 (23%)	6 (19%)	*22 (71%)*

bone marrow probes. Stage IVb patients (n = 36) suffered, in four cases, from a peritoneal carcinosis, which was in accordance with immunocytological positive results in two patients.

Table 1 summarizes the tumor stages, types of resection, and immunocytological results. In 53 patients, peritoneal cavity and bone marrow samples were investigated in pairs. Both compartments were positive in nine patients (17%), including one patient with stage II, one patient with stage III, and seven patients with stage IV (Table 2) tumors.

Follow-Up. The postoperative survival rate was determined in 80 patients and correlated with the findings of 71 bone marrow, 62 peritoneal lavage samples, and 53 pairs (peritoneal lavage plus bone marrow). Due to the majority of patients who died within the first year, the median observation time was short (10.7 months; range, 2–61 months), with an overall 5-year survival rate of 14%.

According to the Kaplan-Meier calculation, all patients with positive immunocytological findings in the peritoneal cavity and in the bone marrow died within 15 and 20 months, respectively. In contrast, 29% of the patients with negative findings (n = 38) were supposed to survive at least 5 years.

The log-rank test showed significance for the peritoneal cavity (P = 0.0035) and a statistical trend for bone marrow findings (P = 0.06). The calculation became highly significant (P < 0.0001) when the immunocytological results of all 80 patients were correlated with the survival (minimal residual disease in either compartment; Fig. 2).

Interestingly, the pair analysis of 53 patients showed that the survival rate of patients with tumor cell dissemination in both compartments (n = 9) was as bad as that in patients who suffered from isolated tumor cells in either the bone marrow (n = 13) or the peritoneal cavity (n = 12; Fig. 3). The detection of micrometastatic single cells strongly correlated in any group with a worse survival rate compared to patients who had no signs of dissemination (n = 19; P = 0.0012). However, the data also strongly indicate that the combined evaluation of bone marrow and peritoneal cavity increases the number of positive patients. This is mainly due to a significant group of patients in whom only one compartment could be analyzed (Table 2).

A prognostic value of immunocytologically detected tumor cells was also observed within patients who suffered from the same tumor stage. A significant correlation was found in stage III when minimal residual disease was detected either in the peritoneal cavity or the bone marrow (P = 0.0216). It is remarkable that, in this advanced tumor stage, the 3-year survival rate in negative patients was 20%, whereas all positive patients except one died within 11 months. The evaluation of R0-resected stage III cases (n = 13) showed that three of four patients (75%) with positive findings in the peritoneal cavity or the bone marrow died within 1 year, compared to four of nine negative patients (44%).

In stage IV, similar results were found: all positive patients died within 20 months, but 12% survived more than 3 years (P = 0.09; Fig. 4). This statistical trend became significant when only lavage samples were calculated (P = 0.0273). Due to the low number of cases with early stages I and II, a comparative statistical calculation of survival and immunocytology for these early stages was not possible. However, when patients with stage I and II were combined, one of three patients with positive immunocytology died within 1 year, in contrast to one of nine patients with negative staining, indicating that the detection of isolated tumor cells also serve as an prognostic factor in early cancer stages.

Additionally, we were interested in the prognostic value of each antibody alone. A highly significant correlation with the survival and peritoneal cavity findings was found for C1P83 (P < 0.0001). Prognostically relevant cells were also found with CA19-9 (P = 0.0224), Ra96 (P = 0.04), and 17-1-A (P = 0.0064). Only the antibody C54-0 did not show a significant correlation (P = 0.1242).

In bone marrow samples, Ra96-positive patients had a significant worse prognosis (P = 0.0324). Although CA19-9 showed a statistical trend (P = 0.1115), none of the other

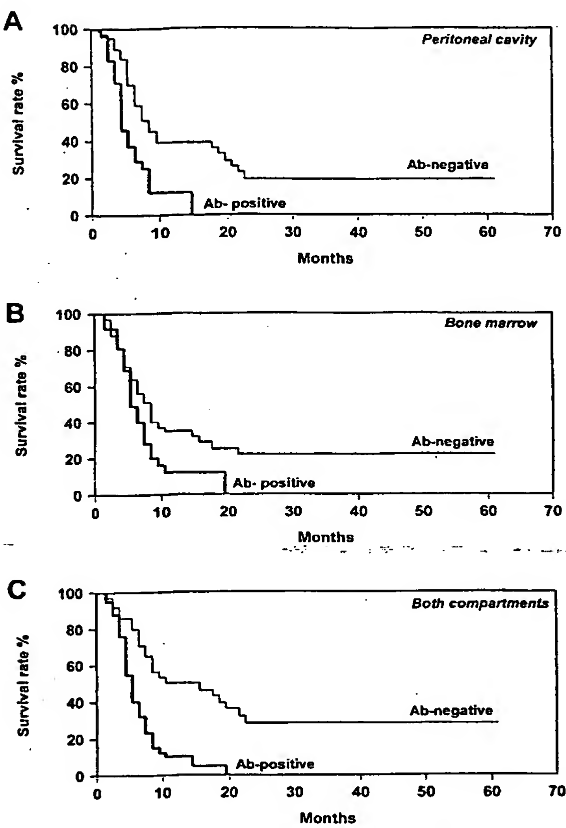

Fig. 2 Kaplan-Meier calculation for the cumulative 5-year survival of pancreatic cancer patients ($n = 80$) with antibody-positive (*Ab-positive*) *versus* antibody-negative (*Ab-negative*) samples. *A*, results of peritoneal lavage samples (*Ab-positive*, $n = 24$; *Ab-negative*, $n = 38$; $P = 0.0035$). *B*, the evaluation of bone marrow samples (*Ab-positive*, $n = 27$; *Ab-negative*, $n = 44$; $P = 0.06$). *C*, results of positive findings in at least one compartment (*Ab-positive*, $n = 42$; *Ab-negative*, $n = 38$; $P < 0.0001$).

antibodies could define a significant correlation of positive immunocytology and survival.

DISCUSSION

Immunocytological techniques have made it possible to detect disseminated tumor cells in the bone marrow of various cancer patients. Most studies have been performed with breast cancer patients using a specific antibody for epithelial cells to detect a tumor cell spread in the bone marrow at the time of operation (23). A strong correlation between tumor cell detection and survival could be seen, and hence, in these patients, the finding of isolated cancer cells may serve as a new prognostic marker (7). Further studies were published describing a similar approach to search for isolated tumor cells in the bone marrow of lung cancer (8), prostatic cancer (10), and neuroblastoma (9) patients. However, in contrast to the mentioned malignancies, bone metastases are a rare event in pancreatic cancer; the majority of the patients suffer from intraabdominal spread (15) and peritoneal carcinosis (24).

Therefore, we investigated peritoneal cavity samples of pancreatic cancer patients in addition to bone marrow aspirates by an immunocytological approach. Previously, we showed that our approach allows a highly specific tumor cell detection in the bone marrow and in the peritoneal cavity (13). This finding was confirmed in this study. The enlarged control group contained only two positive bone marrow and three positive peritoneal lavage samples. Two of these patients were treated by a Whipple resection due to the strong suspicion of a pancreatic cancer. A third patient suffered from chronic hepatitis C and liver cirrhosis and showed lavage cells that were positive for C54-0. The chance of nonspecific mesothelial cell staining by antibodies directed against tumor-associated antigens has been described (25), but obviously, it is low with the applied antibody panel. Additionally, a further explanation for the detection of disseminated cells in the control group might be that those cells are "disseminated" benign cells. This theory receives some supporting evidence from PCR studies. It was found that normal liver cells were detectable in blood samples of patients who were surgically treated for benign liver disease (26). Using a CK20 nested reverse transcription-PCR, which detects disseminated epithelial cancer cells with a high specificity in the bone marrow of colorectal cancer patients, we (27) found, in rare cases with nonmalignant disease, disseminated epithelial cells in bone marrow and blood samples. Interestingly, one of the "control patients" with a CK20-positive bone marrow sample mentioned in that study is identical to one patient in our study who showed CA19-9-stained cells in the bone marrow. The finding of disseminated cells by two different approaches strongly suggests that no nonspecific cross-reaction with normal bone marrow cells occurred.

However, in our study, the finding of disseminated cells without a proven malignancy was a rare event, and overall, this approach was defined to be highly specific for the detection of isolated tumor cells in pancreatic cancer patients. The specificity of the immunocytological results was supported by the observation that, in most tumor patients, at least two different tumor-associated antigens were stained.

Disseminated tumor cells were i.p. found, even in the 20% of patients with an early tumor stage II in whom a direct tumor access to the peritoneal cavity could be excluded. Because studies in gastric cancer patients suggest those cells most likely reach the peritoneum by pores and lymph vessels and obviously become frequently detectable with high sensitive methods such as immunocytology (13, 28). By finding minimal residual disease in peritoneal lavage and bone marrow samples even in early stages, our study give strong evidence that tumor cell spread is a general and an early feature of pancreatic cancer.

Whether isolated disseminated tumor cells possess the ability to form metastatic disease and are, therefore, of prognostic significance is still controversial. Immunocytological studies concerning the predictive value of isolated gastrointestinal tumor cells exist for gastric, colorectal, and esophageal cancer. All studies suggest isolated tumor cells to be a prognostic factor (16, 29–31). Thus far, only one study investigated the prognostic value of epithelial cells in the bone marrow of pancreatic cancer, but the number of patients in that study was low, and therefore, the follow-up data were not conclusive (32).

Our study is the first comprehensive analysis on pancreatic cancer patients. We clearly showed a prognostic dependence of the survival from minimal residual disease. In accordance to recent

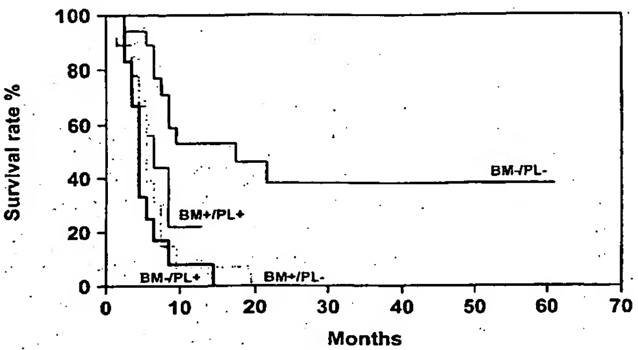

Fig. 3 Correlation of the postoperative survival and immunocytological findings in patients who could be tested in both compartments. Shown are the Kaplan-Meier calculation for patients with no findings in both compartments (*BM−/PL−*, $n = 19$); findings in either the bone marrow (*BM+/PL−*, $n = 13$) or the peritoneal cavity (*BM−/PL+*, $n = 12$); and patients who showed tumor cell dissemination in peritoneal lavage and bone marrow samples (*BM+/PL+*, $n = 9$; $P = 0.0012$).

results in gastric and colorectal cancer (16), the predictive value of i.p. cells was superior to findings in the bone marrow. Interestingly, the pair analysis of 53 patients (Fig. 3) strongly indicates that the predictive value of i.p. isolated tumor cells is not improved by an additional finding of cells in the bone marrow. However, due to a significant number of patients (in our study, almost 20%) in whom a lavage could not be performed, the investigation of the bone marrow helped to increase the number of patients with a prognostically significant minimal residual disease. The higher prognostic value of i.p. tumor cells is not fully understood, but it may be that the contact of tumor cells with peritoneal cells support their ability to develop the full metastatic phenotype (*e.g.*, by secreted growth factors). Gastrointestinal epithelial cells are displaced in the bone marrow and may be in the "wrong" environment and, consequently, kept in a dormant state, as Pantel *et al.* (33) suggested. Further studies will focus on the characterization of the isolated cancer cells to elucidate local factors that may be important in the progress of metastatic disease.

The metastatic potential of isolated tumor cells and, thereby, their prognostic impact became even more evident when patients with the same tumor stage were compared. A Kaplan-Meier calculation was possible for stages III and IV. In both stages, a positive immunocytological result in lavage and/or bone marrow probes significantly correlated with the survival. It is remarkable that all positive patients in stage III and in stage IV died within 18 months, but even in stage IV, some negative patients survived at least 3 years. Furthermore, in stage III patients who received a curative R0 resection, three of four (75%) died within 1 year when minimal residual disease was detected. In contrast, only four of nine (44%) negative patients died within this period.

Due to the low number of positive patients, in stage I and II, a Kaplan-Meier calculation was not performed, but the trend in these early tumor stages was similar to stage III and IV: one of three patients with positive findings died within 1 year. In contrast, one of nine patients in the negative group died during this period from tumor relapse (5-year survival time, 70%; data not shown).

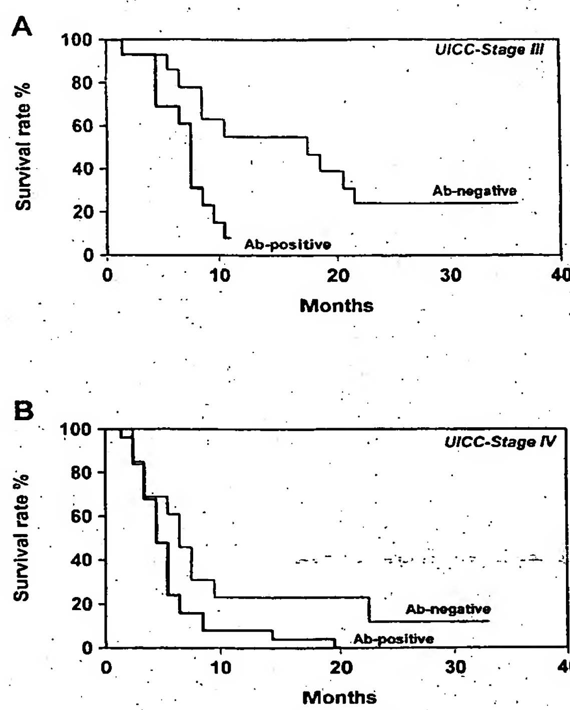

Fig. 4 Correlation of the postoperative survival and immunocytological findings in the peritoneal cavity and/or bone marrow of pancreatic cancer (stage III/IV). Patients with tumor stage III (*Ab-positive*, $n = 9$; *Ab-negative*, $n = 10$; $P = 0.0216$; *A*) and patients with stage IV (*Ab-positive*, $n = 30$; *Ab-negative*, $n = 19$; $P = 0.0809$; *B*) are shown. In stage IV, the statistical evaluation became significant, when only lavage results were evaluated ($P = 0.0273$; data not shown).

By using a significantly higher sensitive technique, our study gives strong support to a recent cytological study that showed a worse prognosis of pancreatic cancer patients suffering from an early i.p. dissemination (34) and can have direct clinical implications: patients who are immunocytologically tested negative might benefit from a more aggressive surgical approach, which is currently not recommended in most stage III and IV patients. On the other hand, immunocytologically positive tested patients, especially patients with tumor cells, have an extremely poor prognosis. A radical surgical approach with a risk of high morbidity seems to be legitimate only if, postoperatively, an effective (and systemic) adjuvant therapy can be offered in the future.

In summary, using an immunocytological approach, we demonstrated that minimal residual disease becomes frequently detectable in the peritoneal cavity and the bone marrow of pancreatic cancer patients. The occurrence of isolated tumor cells correlates with a poor prognosis and, thereby, serves as a new prognostic marker. This technique might be helpful in guiding surgical therapy and new adjuvant treatment concepts.

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Disseminated Tumor Cells in Pancreatic Cancer Patients Detected by Immunocytology: A New Prognostic Factor[1]

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ABSTRACT

Using an immunocytological approach, we previously showed that disseminated cancer cells are frequently found in peritoneal cavity and bone marrow samples of gastrointestinal and pancreatic cancer patients. Recently, we demonstrated that the detection of isolated tumor cells could serve as a new prognostic factor in gastric and colorectal cancer. Thus far, no conclusive data concerning the clinical implication of minimal residual disease in pancreatic cancer exist. In this study, we investigated peritoneal lavage and bone marrow samples of 80 pancreatic cancer patients to determine the predictive value of immunocytologically detected disseminated tumor cells. Therefore, immunocytological findings were correlated with the clinical follow-up data (median observation time, 10.7 months; range, 2–61 months), and the findings in peritoneal cavity and bone marrow samples were compared. Fifty-two % of the patients showed minimal residual disease at least in one compartment (39% positive lavage and 38% positive bone marrow samples). The detection rate of isolated tumor cells increased in parallel to the tumor stage. The presence of tumor cells in the peritoneal cavity significantly correlated with the survival time of the patients ($P = 0.0035$). In bone marrow samples, a strong trend was seen ($P = 0.06$). The evaluation of both compartments increased the number of positive patients and resulted in a highly significant correlation: all patients who were positive in at least one compartment died within 18 months, whereas negative patients showed a 5-year survival rate of 30% ($P < 0.0001$). We recommend immunocytological investigation of peritoneal cavity and bone marrow samples as a new prognostic marker in pancreatic cancer patients.

INTRODUCTION

The prognosis of pancreatic cancer is extremely poor; the overall 5-year survival rate is only 2%. A chance of cure exists only for a minority of patients with locally limited and surgically resectable tumors (1). However, of patients who are radically treated by a partial pancreatico-duodenectomy and lymphadenectomy (R0 resection), 70–80% will suffer from an incurable local relapse, distant metastases, or peritoneal carcinosis (2). Although a local relapse might be caused by an incomplete resection, distant metastases and peritoneal carcinosis ultimately depend on a dissemination of malignant cells. Presently, those cells are not detectable with conventional diagnostic tools. However, their elimination is the aim of various adjuvant therapy concepts that are currently under investigation, including chemotherapy (3), immunotherapy (4), and gene therapy (5). For a specific selection of patients who would benefit from those therapies, it would be helpful to detect minimal residual disease.

Immunocytological methods, which are significantly more sensitive than conventional cytology (6), have made it possible to detect disseminated tumor cells in the bone marrow of patients with breast cancer (7), small cell lung cancer (8), neuroblastoma (9), prostatic cancer (10), gastric cancer (11), and colorectal cancer (12). Previously, we could demonstrate that minimal residual disease also frequently occurs in bone marrow of pancreatic cancer patients (13, 14). However, bone marrow metastases are a rare event in pancreatic cancer (15). Disseminated tumor cells are more likely to occur in the peritoneal cavity because of the high frequency of local relapse or peritoneal carcinosis. Therefore, we also developed an immunocytological approach to detect isolated tumor cells in peritoneal cavity samples (13). Recently, we showed that the finding of tumor cells in the peritoneal cavity can serve as a prognostic marker in gastric and colorectal cancer (16). However, no comprehensive immunocytological studies exist concerning the clinical implication of isolated i.p. and bone marrow tumor cells in pancreatic cancer.

This study was initiated to determine the predictive value of disseminated tumor cells in pancreatic cancer patients. We extended our former studies to a larger collection of patients and investigated peritoneal lavage and, for comparison, bone marrow samples in surgically treated tumor patients and compared the immunocytological findings with the clinical data.

Here, we show that tumor cells were frequently detectable in the peritoneal cavity and in the bone marrow. A finding of isolated tumor cells correlated significantly with the postoperative survival rate of pancreatic cancer patients.

MATERIALS AND METHODS

Patients

All patients were extensively informed and gave written consent for the investigations, including the bone marrow aspi-

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ration. The study was confirmed by the ethics commission of the University Hospital Kiel (Kiel, Germany).

We evaluated the data of 80 patients who suffered from an adenocarcinoma of the pancreas and underwent surgery (carcinoma of the papilla vateri and endocrine tumors were excluded). Bone marrow samples were tested in 71 patients (9 patients declined to give their consent), and lavage samples could be obtained in 62 patients (18 patients showed adhesions that were too extensive). Pairs of peritoneal lavage and bone marrow were analyzed in 53 patients.

Patients who died in the hospital due to surgical complications or tumor progress (n = 7) and patients who attended a clinical study for adjuvant therapy (n = 2) were excluded from this study. Another six patients who were initially investigated were lost in the follow-up and, therefore, not included in this study (1 stage II and positive in peritoneal lavage; 2 stage III and immunocytologically negative; and 3 stage IV, 2 of which were immunocytologically positive).

Control Group. Fifty-eight patients who underwent surgery but did not suffer from a malignant disease served as a control group; 45 agreed to a bone marrow aspiration and 43 agreed to a peritoneal lavage. The control group included patients suffering from benign liver tumors (n = 10), sigma diverticulitis (n = 8), chronic pancreatitis (n = 7), cholecystolithiasis (n = 5), achalasia (n = 4), other benign diseases (n = 3), and hypersplenism (n = 4). Additionally, ascites from patients with liver cirrhosis (n = 5) and bone marrow samples from patients with benign hematological disease (n = 12) were examined.

Samples

Bone marrow (10 ml) was aspirated from the right spina iliaca anterior at the beginning of the operation (Jamshidi needle). Peritoneal lavage was performed before manipulation of the tumor. One liter of isotonic sodium chloride solution was instilled and immediately removed (13).

Preparation of Samples. The lavage solution was centrifuged (270 × g for 10 min), and the supernatant was discharged. The cell pellet was resuspended in 20 ml of RPMI 1640. All lavage suspensions and bone marrow aspirates were further processed by Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged onto microscopic slides (2.5 × 10^5 cells/slide). Cytospins were fixed in acetone and stored at −20°C (13).

Immunocytochemistry

Staining of cytospins was performed by the immunoperoxidase method with six different monoclonal antibodies as described previously (13): (*a*) C1P83, gold 3 epitope of CEA[3] (17); (*b*) CA19-9 (DAKO, Carpinteria, CA), determinants of Lewis blood group antigens (18); (*c*) 17-1-A (Centocor, Leiden, the Netherlands), tumor-associated membrane antigen (19); (*d*) Ra96, tumor-associated mucin antigen (20); (*e*) C54-0, epithelial membrane antigen (21): because of a cross-reactivity with a subpopulation of lymphopoietic cells in the bone marrow, C54-0 was only evaluated for peritoneal cavity samples; and (*f*) KL-1 (Immunotech, Hamburg, Germany), cytokeratins (only used in bone marrow samples). The hybridoma cells for the antibodies C1P83, Ra96, and C54-0 were produced by our laboratory, and antibodies were purified according to standard procedures (21). A total of 1.25 × 10^6 peritoneal lavage and bone marrow cells each were tested for every patient. The microscopic evaluation was carried out independently by two investigators. To ensure that the investigators were not influenced by the clinical data, the slides were blinded by numbers, and the investigators were, therefore, not aware of the clinical background of the samples (*e.g.*, tumor stage, control sample, and so on).

Evaluation of Data

Samples were evaluated as tumor cell positive if at least one cell reacted with one of the monoclonal antibodies. The detection rate was correlated with the Union International Contre Cancer classification of the tumor stage and the R-classification (22).

Postoperatively, patients were examined either in our outpatient clinic or by their general practitioner. Every 3 months, a clinical examination and blood tests, including tumor markers CEA and CA19-9, were performed, and every 6 months, a sonography or computed tomography scan was performed.

The projected survival rates were determined by Kaplan-Meier calculation, and calculations of significance were determined by the log-rank test.

RESULTS

Control Group. In 43 of 45 patients, no cell staining was seen in bone marrow samples with the antibodies KL-1, C1P83, Ra96, CA19-9, or 17-1-A. In two patients, single cells were stained with the antibodies KL-1, C1P83, and 17-1-A. Additionally, one patient had positive cells for CA19-9. Both patients were strongly suspected to suffer from pancreatic cancer and were, therefore, treated by a partial duodenopancreatectomy (Whipple operation). The histological analysis could not confirm this diagnosis and found a chronic pancreatitis.

The peritoneal lavage samples of patients with no malignant diseases showed, in 40 of 43 cases, no positive reaction of the applied antibodies (C1P83, Ra96, CA19-9, 17-1-A, and C54-0). Two of the positive samples were derived from the same patients mentioned above who suffered from a chronic pancreatitis and showed C54-0- and C1P8-stained i.p. cells, respectively. A third patient with liver cirrhosis and a chronic hepatitis C was positive for the antibody C54-0.

Pancreatic Cancer. Overall, minimal residual disease was detected in 42 of 80 (52%) patients; 24 of 62 (39%) had positive peritoneal cavities and 27 of 71 (38%) had positive bone marrow samples. The antibody CA19-9 showed the highest detection rate in bone marrow samples (23%), followed by C1P83 (anti-CEA) and KL-1 (13 and 12%, respectively). In peritoneal cavity samples, C1P83 (31%) and Ra96 (20%) reacted with the highest frequency. As shown previously (13), the combination of all antibodies clearly increased the detection rate in the bone marrow, peritoneal cavity, and combined evaluation (38, 39, and 52%, respectively). On average, we found 5 isolated tumor cells per peritoneal lavage sample (range, 1–95 cells) and 3 isolated tumor cells in the bone marrow (range, 1–5 cells).

[3] The abbreviation used is: CEA, carcinoembryonic antigen.

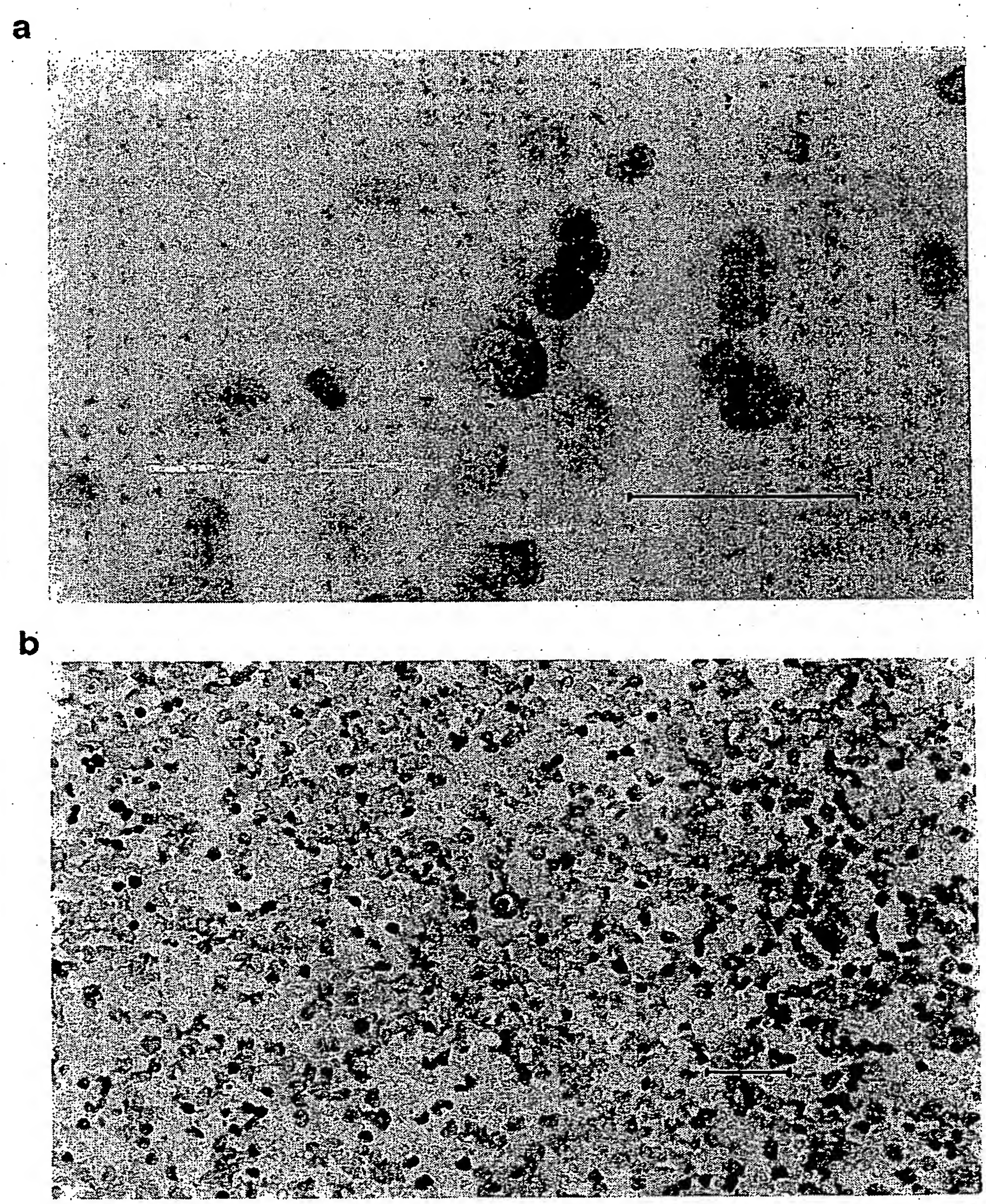

Fig. 1 Cytospins of a peritoneal lavage sample from a pancreatic cancer patient (*a*) and of a bone marrow sample (*b*) stained with CA19-9 and KL-1, respectively (*scale bar*, 50 μm).

Fig. 1 shows typical positive results in a peritoneal lavage (Fig. 1*a*) and a bone marrow sample (Fig. 1*b*). In peritoneal cavity and bone marrow samples, the detection rate increased in parallel with the tumor stage. Interestingly, one of five (20%) patients with a stage II tumor showed positive cells within the peritoneal cavity, although the tumor had no direct access to the peritoneal cavity; three of eight (38%) presented tumor cells in the bone marrow; and in three of nine (33%), the tumor had spread in both compartments.

A radical partial pancreatico-duodenectomy (Whipple procedure) including extended lymphadenectomy was performed as an R0 resection in 29 patients. In 6 patients, the histological evaluation revealed a R1 resection (microscopically remaining tumor); in 3 patients, a macroscopically incomplete tumor resection was performed (R2 resection); and 42 patients received palliative surgery by abdominal exploration and bypass operation (*e.g.*, gastroenterostomy and biliodigestive anastomosis).

In R0-resected patients, minimal residual disease was detected in 29% of the peritoneal cavity and 21% of the bone marrow samples. In incomplete resected or palliative treated patients, the detection rate was significantly higher and showed tumor cell dissemination in 56% of the peritoneal cavity and in 48% of the

Table 1 Summary of pancreatic cancer patients ($n = 80$) who were included in the follow-up study

Shown are the Union International Contre Cancer tumor stage; the number of patients with a "curative" operation (R0 resection), incomplete resection (R1/2 resection), or palliative operation (OP); and the detection rate in the peritoneal cavity (pc), bone marrow (bm), or either compartment (pc and/or bm).

Stage	R0 resection ($n = 29$)	R1/2 resection ($n = 9$)	Palliative OP ($n = 42$)	Detection rate		
				pc	bm	pc and/or bm
I	3	0	0	0/2 (0%)	0/3 (0%)	0/3 (0%)
II	8	1	0	1/5 (20%)	3/8 (38%)	3/9 (33%)
III	13	0	6	3/13 (23%)	7/16 (44%)	9/19 (47%)
IVa	2	4	3	3/6 (50%)	3/9 (33%)	5/9 (56%)
IVb	3	4	33	17/36 (47%)	14/35 (40%)	25/40 (63%)

Table 2 Summary of 53 patients in whom peritoneal lavage and bone marrow were investigated in parallel ("pairs")

Comparison of tumor stage and number of patients (percentages in parentheses) with positive peritoneal cavity samples only (pc only), positive bone marrow samples only (bm only), patients with immunocytological findings in both compartments (pc and bm), and patients with positive findings in either compartment (pc and/or bm).

Stage	Detection rate			
	pc only	bm only	pc and bm	pc and/or bm
I ($n = 2$)	0 (0%)	0 (0%)	0 (0%)	*0 (0%)*
II ($n = 4$)	0 (0%)	1 (25%)	1 (25%)	*2 (50%)*
III ($n = 10$)	1 (10%)	4 (40%)	1 (10%)	*6 (60%)*
IVa ($n = 6$)	2 (33%)	1 (17%)	1 (17%)	*4 (66%)*
IVb ($n = 31$)	9 (29%)	7 (23%)	6 (19%)	*22 (71%)*

bone marrow probes. Stage IVb patients ($n = 36$) suffered, in four cases, from a peritoneal carcinosis, which was in accordance with immunocytological positive results in two patients.

Table 1 summarizes the tumor stages, types of resection, and immunocytological results. In 53 patients, peritoneal cavity and bone marrow samples were investigated in pairs. Both compartments were positive in nine patients (17%), including one patient with stage II, one patient with stage III, and seven patients with stage IV (Table 2) tumors.

Follow-Up. The postoperative survival rate was determined in 80 patients and correlated with the findings of 71 bone marrow, 62 peritoneal lavage samples, and 53 pairs (peritoneal lavage plus bone marrow). Due to the majority of patients who died within the first year, the median observation time was short (10.7 months; range, 2–61 months), with an overall 5-year survival rate of 14%.

According to the Kaplan-Meier calculation, all patients with positive immunocytological findings in the peritoneal cavity and in the bone marrow died within 15 and 20 months, respectively. In contrast, 29% of the patients with negative findings ($n = 38$) were supposed to survive at least 5 years.

The log-rank test showed significance for the peritoneal cavity ($P = 0.0035$) and a statistical trend for bone marrow findings ($P = 0.06$). The calculation became highly significant ($P < 0.0001$) when the immunocytological results of all 80 patients were correlated with the survival (minimal residual disease in either compartment; Fig. 2).

Interestingly, the pair analysis of 53 patients showed that the survival rate of patients with tumor cell dissemination in both compartments ($n = 9$) was as bad as that in patients who suffered from isolated tumor cells in either the bone marrow ($n = 13$) or the peritoneal cavity ($n = 12$; Fig. 3). The detection of micrometastatic single cells strongly correlated in any group with a worse survival rate compared to patients who had no signs of dissemination ($n = 19$; $P = 0.0012$). However, the data also strongly indicate that the combined evaluation of bone marrow and peritoneal cavity increases the number of positive patients. This is mainly due to a significant group of patients in whom only one compartment could be analyzed (Table 2).

A prognostic value of immunocytologically detected tumor cells was also observed within patients who suffered from the same tumor stage. A significant correlation was found in stage III when minimal residual disease was detected either in the peritoneal cavity or the bone marrow ($P = 0.0216$). It is remarkable that, in this advanced tumor stage, the 3-year survival rate in negative patients was 20%, whereas all positive patients except one died within 11 months. The evaluation of R0-resected stage III cases ($n = 13$) showed that three of four patients (75%) with positive findings in the peritoneal cavity or the bone marrow died within 1 year, compared to four of nine negative patients (44%).

In stage IV, similar results were found: all positive patients died within 20 months, but 12% survived more than 3 years ($P = 0.09$; Fig. 4). This statistical trend became significant when only lavage samples were calculated ($P = 0.0273$). Due to the low number of cases with early stages I and II, a comparative statistical calculation of survival and immunocytology for these early stages was not possible. However, when patients with stage I and II were combined, one of three patients with positive immunocytology died within 1 year, in contrast to one of nine patients with negative staining, indicating that the detection of isolated tumor cells also serve as an prognostic factor in early cancer stages.

Additionally, we were interested in the prognostic value of each antibody alone. A highly significant correlation with the survival and peritoneal cavity findings was found for C1P83 ($P < 0.0001$). Prognostically relevant cells were also found with CA19-9 ($P = 0.0224$), Ra96 ($P = 0.04$), and 17-1-A ($P = 0.0064$). Only the antibody C54-0 did not show a significant correlation ($P = 0.1242$).

In bone marrow samples, Ra96-positive patients had a significant worse prognosis ($P = 0.0324$). Although CA19-9 showed a statistical trend ($P = 0.1115$), none of the other

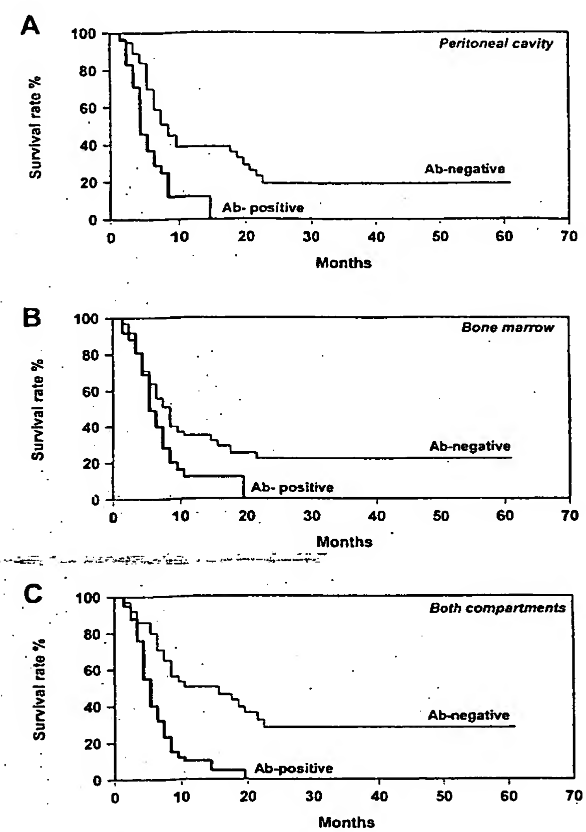

Fig. 2 Kaplan-Meier calculation for the cumulative 5-year survival of pancreatic cancer patients ($n = 80$) with antibody-positive (*Ab-positive*) *versus* antibody-negative (*Ab-negative*) samples. *A*, results of peritoneal lavage samples (*Ab-positive*, $n = 24$; *Ab-negative*, $n = 38$; $P = 0.0035$). *B*, the evaluation of bone marrow samples (*Ab-positive*, $n = 27$; *Ab-negative*, $n = 44$; $P = 0.06$). *C*, results of positive findings in at least one compartment (*Ab-positive*, $n = 42$; *Ab-negative*, $n = 38$; $P < 0.0001$).

antibodies could define a significant correlation of positive immunocytology and survival.

DISCUSSION

Immunocytological techniques have made it possible to detect disseminated tumor cells in the bone marrow of various cancer patients. Most studies have been performed with breast cancer patients using a specific antibody for epithelial cells to detect a tumor cell spread in the bone marrow at the time of operation (23). A strong correlation between tumor cell detection and survival could be seen, and hence, in these patients, the finding of isolated cancer cells may serve as a new prognostic marker (7). Further studies were published describing a similar approach to search for isolated tumor cells in the bone marrow of lung cancer (8), prostatic cancer (10), and neuroblastoma (9) patients. However, in contrast to the mentioned malignancies, bone metastases are a rare event in pancreatic cancer; the majority of the patients suffer from intraabdominal spread (15) and peritoneal carcinosis (24).

Therefore, we investigated peritoneal cavity samples of pancreatic cancer patients in addition to bone marrow aspirates by an immunocytological approach. Previously, we showed that our approach allows a highly specific tumor cell detection in the bone marrow and in the peritoneal cavity (13). This finding was confirmed in this study. The enlarged control group contained only two positive bone marrow and three positive peritoneal lavage samples. Two of these patients were treated by a Whipple resection due to the strong suspicion of a pancreatic cancer. A third patient suffered from chronic hepatitis C and liver cirrhosis and showed lavage cells that were positive for C54-0. The chance of nonspecific mesothelial cell staining by antibodies directed against tumor-associated antigens has been described (25), but obviously, it is low with the applied antibody panel. Additionally, a further explanation for the detection of disseminated cells in the control group might be that those cells are "disseminated" benign cells. This theory receives some supporting evidence from PCR studies. It was found that normal liver cells were detectable in blood samples of patients who were surgically treated for benign liver disease (26). Using a CK20 nested reverse transcription-PCR, which detects disseminated epithelial cancer cells with a high specificity in the bone marrow of colorectal cancer patients, we (27) found, in rare cases with nonmalignant disease, disseminated epithelial cells in bone marrow and blood samples. Interestingly, one of the "control patients" with a CK20-positive bone marrow sample mentioned in that study is identical to one patient in our study who showed CA19-9-stained cells in the bone marrow. The finding of disseminated cells by two different approaches strongly suggests that no nonspecific cross-reaction with normal bone marrow cells occurred.

However, in our study, the finding of disseminated cells without a proven malignancy was a rare event, and overall, this approach was defined to be highly specific for the detection of isolated tumor cells in pancreatic cancer patients. The specificity of the immunocytological results was supported by the observation that, in most tumor patients, at least two different tumor-associated antigens were stained.

Disseminated tumor cells were i.p. found, even in the 20% of patients with an early tumor stage II in whom a direct tumor access to the peritoneal cavity could be excluded. Because studies in gastric cancer patients suggest those cells most likely reach the peritoneum by pores and lymph vessels and obviously become frequently detectable with high sensitive methods such as immunocytology (13, 28). By finding minimal residual disease in peritoneal lavage and bone marrow samples even in early stages, our study give strong evidence that tumor cell spread is a general and an early feature of pancreatic cancer.

Whether isolated disseminated tumor cells possess the ability to form metastatic disease and are, therefore, of prognostic significance is still controversial. Immunocytological studies concerning the predictive value of isolated gastrointestinal tumor cells exist for gastric, colorectal, and esophageal cancer. All studies suggest isolated tumor cells to be a prognostic factor (16, 29–31). Thus far, only one study investigated the prognostic value of epithelial cells in the bone marrow of pancreatic cancer, but the number of patients in that study was low, and therefore, the follow-up data were not conclusive (32).

Our study is the first comprehensive analysis on pancreatic cancer patients. We clearly showed a prognostic dependence of the survival from minimal residual disease. In accordance to recent

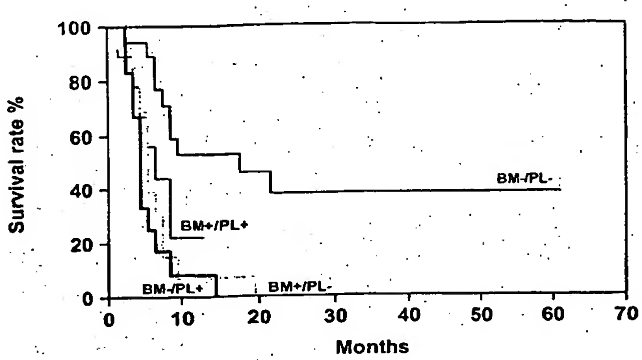

Fig. 3 Correlation of the postoperative survival and immunocytological findings in patients who could be tested in both compartments. Shown are the Kaplan-Meier calculation for patients with no findings in both compartments (*BM−/PL−*, $n = 19$); findings in either the bone marrow (*BM+/PL−*, $n = 13$) or the peritoneal cavity (*BM−/PL+*, $n = 12$); and patients who showed tumor cell dissemination in peritoneal lavage and bone marrow samples (*BM+/PL+*, $n = 9$; $P = 0.0012$).

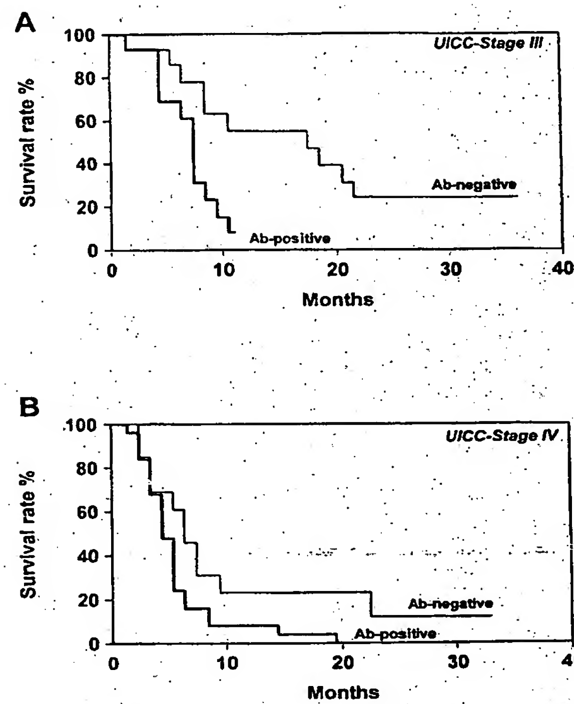

Fig. 4 Correlation of the postoperative survival and immunocytological findings in the peritoneal cavity and/or bone marrow of pancreatic cancer (stage III/IV). Patients with tumor stage III (*Ab-positive*, $n = 9$; *Ab-negative*, $n = 10$; $P = 0.0216$; *A*) and patients with stage IV (*Ab-positive*, $n = 30$; *Ab-negative*, $n = 19$; $P = 0.0809$; *B*) are shown. In stage IV, the statistical evaluation became significant, when only lavage results were evaluated ($P = 0.0273$; data not shown).

results in gastric and colorectal cancer (16), the predictive value of i.p. cells was superior to findings in the bone marrow. Interestingly, the pair analysis of 53 patients (Fig. 3) strongly indicates that the predictive value of i.p. isolated tumor cells is not improved by an additional finding of cells in the bone marrow. However, due to a significant number of patients (in our study, almost 20%) in whom a lavage could not be performed, the investigation of the bone marrow helped to increase the number of patients with a prognostically significant minimal residual disease. The higher prognostic value of i.p. tumor cells is not fully understood, but it may be that the contact of tumor cells with peritoneal cells support their ability to develop the full metastatic phenotype (*e.g.*, by secreted growth factors). Gastrointestinal epithelial cells are displaced in the bone marrow and may be in the "wrong" environment and, consequently, kept in a dormant state, as Pantel *et al.* (33) suggested. Further studies will focus on the characterization of the isolated cancer cells to elucidate local factors that may be important in the progress of metastatic disease.

The metastatic potential of isolated tumor cells and, thereby, their prognostic impact became even more evident when patients with the same tumor stage were compared. A Kaplan-Meier calculation was possible for stages III and IV. In both stages, a positive immunocytological result in lavage and/or bone marrow probes significantly correlated with the survival. It is remarkable that all positive patients in stage III and in stage IV died within 18 months, but even in stage IV, some negative patients survived at least 3 years. Furthermore, in stage III patients who received a curative R0 resection, three of four (75%) died within 1 year when minimal residual disease was detected. In contrast, only four of nine (44%) negative patients died within this period.

Due to the low number of positive patients, in stage I and II, a Kaplan-Meier calculation was not performed, but the trend in these early tumor stages was similar to stage III and IV: one of three patients with positive findings died within 1 year. In contrast, one of nine patients in the negative group died during this period from tumor relapse (5-year survival time, 70%; data not shown).

By using a significantly higher sensitive technique, our study gives strong support to a recent cytological study that showed a worse prognosis of pancreatic cancer patients suffering from an early i.p. dissemination (34) and can have direct clinical implications: patients who are immunocytologically tested negative might benefit from a more aggressive surgical approach, which is currently not recommended in most stage III and IV patients. On the other hand, immunocytologically positive tested patients, especially patients with tumor cells, have an extremely poor prognosis. A radical surgical approach with a risk of high morbidity seems to be legitimate only if, postoperatively, an effective (and systemic) adjuvant therapy can be offered in the future.

In summary, using an immunocytological approach, we demonstrated that minimal residual disease becomes frequently detectable in the peritoneal cavity and the bone marrow of pancreatic cancer patients. The occurrence of isolated tumor cells correlates with a poor prognosis and, thereby, serves as a new prognostic marker. This technique might be helpful in guiding surgical therapy and new adjuvant treatment concepts.

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APPENDIX I

Detection of Disseminated Colorectal Cancer Cells in Lymph Nodes, Blood and Bone Marrow

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ABSTRACT

Tumor progression after curative resection of colorectal cancer is caused by tumor cell dissemination, currently undetected by standard clinical staging techniques. The detection of disseminated tumor cells could help to identify a patient subgroup at risk for disease relapse who could benefit from adjuvant therapy. In addition, the significance of lymphogenic compared with hematogenic colorectal cancer cell dissemination is unknown. However, this knowledge would strongly influence the development of future therapeutic regimes. The purpose of this study was to determine the extent of colorectal cancer cell dissemination in lymph nodes compared with blood and bone marrow. Using a CK 20-reverse transcription (RT)-PCR assay, we examined 279 lymph nodes, blood, and bone marrow samples from 20 patients with colorectal cancer. Of 16 patients (11 patients stage I, 5 patients stage II) with histopathologically tumor-free lymph nodes: 14 patients (10 patients stage I, 4 patients stage II) were found to have tumor cells in paracolonic lymph nodes; 12 patients (8 patients stage I, 4 patients stage II) were found to have tumor cells in the lymph nodes along the mesentery vessels; and, remarkably, 6 patients (4 patients stage I, 2 patients stage II) were found to have tumor cells in the apical lymph nodes. In contrast, tumor cells were detected in only two blood and three bone marrow samples of these patients. Thus, lymphogenic tumor cell dissemination is a very common and early event in colorectal cancer, preceding hematogenic tumor cell dissemination. In addition, our data strongly suggest that the detection of tumor cells in the apical lymph node by CK 20-RT-PCR has prognostic relevance. Our results underline the therapeutic importance of meticulous lymph node dissection and demonstrate that the detection of lymphogenic or hematogenic tumor cell dissemination by CK 20-RT-PCR will significantly improve current tumor staging protocols.

INTRODUCTION

Patients with colorectal cancer initially presenting with resectable tumors and tumor-free lymph nodes (UICC[2] stage I or II) are generally considered as patients at low risk for recurrence. Therefore, adjuvant therapy is not recommended in these cases. Despite the low tumor stage, about 30–40% of these patients subsequently develop recurrent disease (1). These findings indicate that hematogenic or lymphogenic dissemination of metastatic tumor cells, not detectable by conventional staging techniques, must have occurred in some of these patients. Detection of disseminated tumor cells in patients with colorectal cancer UICC stage I or II may identify high-risk patients for tumor recurrence, who could benefit from adjuvant therapy regimens. The comparative analysis of lymph nodes, blood, and bone marrow may define the relative incidence of hematogenic *versus* lymphogenic tumor cell dissemination in colorectal cancer. This data could help to optimize strategies for the treatment of colorectal cancer, for example, by defining the relative importance of surgical lymphadenectomy *versus* systemic adjuvant therapy.

Immunocytological methods have been applied to detect hematogenic tumor cell dissemination (2). Tumor cell detection was clearly related to an earlier relapse and decreased survival of the respective patients. RT-PCR-based protocols have further improved the sensitivity and specificity of detection systems for disseminated cancer cells in blood and bone marrow (3–9).

The second route of tumor cell dissemination is via the lymphatic system (10). Because the routine histopathological methods of lymph node examination show limited sensitivity, several techniques have been studied in an attempt to increase the detection of occult lymph node micrometastases, including serial sectioning of lymph nodes (11), immunohistology (12–17), and, finally, PCR-based techniques (18–26). These studies, however, revealed contradictory results regarding the incidence of occult lymph node micrometastases and their prognostic significance.

Thus, the extent and prognostic significance of lymphogenic tumor cell dissemination in colorectal cancer has still not been sufficiently defined. In addition, the relative significance of lymphogenic *versus* hematogenic tumor cell dissemination is

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[2] The abbreviations used are: UICC, International Union Against Cancer; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde phosphate dehydrogenase; CEA, carcinoembryonic antigen.

still a matter of debate (27). The purpose of the present study was to determine the extent of dissemination of colorectal cancer cells in lymph nodes and to correlate these results with the detection of disseminated tumor cells in bone marrow and blood samples of the same patients.

PATIENTS AND METHODS

Lymph Node and Tumor Sampling. Lymph nodes were freshly harvested from the resected specimen. Initially, the mesentery was separated from the gut to prevent any cross-contamination with CK 20 positive cells. Then the lymph nodes were dissected from the mesentery. The location of the lymph nodes was recorded [apical lymph node, lymph node at the central lymphovascular ligation, lymph nodes at the main vessel (for example, inferior mesenteric artery), and paracolonic lymph nodes]. The lymph nodes were then halved; one-half was used for conventional histopathology, and the other half was shock-frozen in liquid nitrogen and stored at −70°C. From the fresh resection specimen, a tumor sample was excised and shock-frozen. Before RNA extraction, frozen tissue sections of tumors and lymph nodes were performed. From each lymph node half, 20 representative sections with a diameter of 20 μm were used for RNA extraction.

Blood and Bone Marrow Samples. After the induction of general anesthesia, 10-ml blood samples were obtained preoperatively through a central venous catheter in the vena cava superior and were diluted with 10 ml of PBS. Bone marrow samples (10-ml) were obtained concurrently by aspiration from both iliac crests. After density centrifugation through Ficoll-Paque (Pharmacia; 30 min, 400 × *g*) mononuclear cells were harvested from the interphase and washed twice in PBS. The cell pellet was then shock-frozen in liquid nitrogen and stored at −70°C.

RNA Extraction. RNA extraction from peripheral mononuclear blood cells and from bone marrow samples and frozen tissue sections of tumors and lymph nodes was performed as described previously (9).

RT-PCR. CK 20-RT-PCR was performed as described previously (9). PCR products were analyzed by electrophoresis on 2% agarose gels. CK 20-PCR products were blotted onto nylon membranes (Hybond N+, Amersham Life Science, Buckinghamshire, United Kingdom) and hybridized with a chemoluminescence-labeled oligonucleotide probe (ECL detection system, Amersham Life Science) comprising nucleotides 5269–5280 on exon 1 and nucleotides 7429–7448 on exon 2 of the CK 20 sequence (CTG CGA AGT CAG ATT AAG GAT G) as recommended by the supplier.

RNA quality and performance of reverse transcription of all of the analyzed samples was confirmed by RT-PCR amplification of GAPDH transcripts as described previously (28).

Patients. Informed consent was obtained from all of the patients. The study protocol was approved by the Ethics Committee of the University of Heidelberg.

Twenty patients treated at the Department of Surgery, University of Heidelberg (13 male, 7 female; ages 48–86, mean 66.4) were included. All of the patients had histologically confirmed colorectal adenocarcinoma (9 rectal carcinoma, 11 colon carcinoma) undergoing curative (R0) resection according to the "no-touch isolation" technique (29) with systematic lymph node dissection. Tumor stage and grading were classified according to the 5th edition of the TNM classification of the UICC (30).

Paracolonic lymph nodes from 22 patients undergoing colorectal resection for benign diseases (sigmoid diverticulosis, Crohn's disease and ulcerative colitis) served as controls.

RESULTS

Specificity of CK 20-RT-PCR

Paracolonic lymph node samples ($n = 22$) from 22 patients undergoing colorectal resection for benign disease were examined for expression of CK 20. None of these samples revealed a detectable CK 20-RT-PCR amplification product. All of the lymph nodes showed a positive GAPDH amplification signal, which confirmed the quality of the RNA and the adequate performance of reverse transcription. The 20 analyzed primary tumor samples all tested positive for CK 20 mRNA expression. Specificity of CK 20-RT-PCR for tumor cell detection in blood and bone marrow has been demonstrated previously (7, 9).

Patient Study

Detection of Disseminated Tumor Cells in Lymph Node Samples of Patients with Colorectal Carcinoma. In a prospective study, we analyzed 279 lymph nodes from 20 patients with colorectal cancer (mean, 13.95 lymph nodes per patient) histopathologically and by CK 20-RT-PCR. Of the 279 examined lymph nodes, 10 were positive by histopathological examination; all of these also revealed a positive CK 20 signal. Using the RT-PCR, 137 (50.9%) of the 269 histopathologically tumor-free lymph nodes revealed CK 20 amplification products.

Four of the 20 patients had positive lymph nodes on routine histopathological examination (stage III), and 16 patients had negative lymph nodes (11 patients stage I, 5 patients stage II). CK 20-RT-PCR revealed one or more positive lymph nodes in 14 of the 16 patients [UICC stage I or II (10 of 11 patients stage I; 4 of 5 patients stage II; Table 1; Fig. 1]. The results were stratified according to lymph node location [apical lymph node, lymph nodes along the main vessel (for example, inferior mesenteric artery), and paracolonic lymph nodes). In the patients with UICC stage I or II tumors, the apical lymph node revealed a positive CK 20 signal in 6 (37.5%) of 16 patients (4 of 11 patients stage I, 2 of 5 patients stage II), the lymph nodes at the main vessel in 12 (75%) of 16 patients (8 of 11 patients stage I, 4 of 5 patients stage II), and the paracolonic lymph nodes in 14 (87.5%) of 16 patients (10 of 11 patients stage I, 4 of 5 patients stage II; Tables 1 and 2). All of the six patients with positive apical lymph node also revealed positive lymph nodes along the main vessel and in the paracolonic tissue. All of the six patients with a negative central lymph node but positive lymph nodes along the main vessel also had positive paracolonic lymph nodes.

Comparative Analysis of Tumor Detection Rates in Lymph Nodes, Venous Blood, and Bone Marrow Samples. Bone marrow samples from 16 patients and preoperative blood samples from 20 patients were analyzed in addition to the lymph nodes. In patients at UICC stage I or II, 3 (21.4%) of 14 bone marrow samples and 2 (12.5%) of 16 blood samples revealed a CK 20 amplification product (Table 2).

Table 1 Results of the detection of tumor cells in lymph nodes by conventional histopathology compared to CK 20-RT-PCR

Patient No	Stage	Conventional histopathology	CK 20-RT-PCR	Location of CK 20-positive nodes: Central	Location of CK 20-positive nodes: Main vessel	Location of CK 20-positive nodes: Paracolonic
1	I	0 of 14	7 of 14	Pos[a]	1 of 4	5 of 9
2	II	0 of 10	0 of 10	Neg	0 of 1	0 of 8
3	I	0 of 13	4 of 13	Neg	0 of 7	4 of 5
4	I	0 of 7	6 of 7	Pos	1 of 2	4 of 4
5	I	0 of 11	4 of 11	Neg	2 of 2	2 of 8
6	I	0 of 12	2 of 12	Neg	0 of 4	2 of 7
7	I	0 of 13	0 of 13	Neg	0 of 4	0 of 8
8	II	0 of 14	9 of 14	Neg	1 of 3	8 of 10
9	II	0 of 15	3 of 15	Neg	1 of 6	2 of 8
10	I	0 of 17	10 of 17	Neg	2 of 4	8 of 12
11	I	0 of 10	4 of 10	Neg	2 of 2	2 of 7
12	I	0 of 19	19 of 19	Pos	11 of 11	7 of 7
13	I	0 of 15	13 of 15	Pos	6 of 6	6 of 8
14	II	0 of 23	16 of 23	Pos	5 of 6	10 of 16
15	II	0 of 24	13 of 24	Pos	3 of 9	9 of 14
16	I	0 of 18	6 of 18	Neg	3 of 9	3 of 8
17	III	3 of 11	7 of 11	Neg	4 of 7	3 of 3
18	III	1 of 9	6 of 9	Neg	5 of 6	1 of 2
19	III	3 of 13	12 of 13	Pos	5 of 6	6 of 6
20	III	3 of 11	6 of 11	Pos	2 of 4	3 of 6
		10 of 279 3.58%	147 of 279 52.7%	8 of 20 40%	54 of 103 52.4%	85 of 156 54.5%

[a] Pos, positive; Neg, negative.

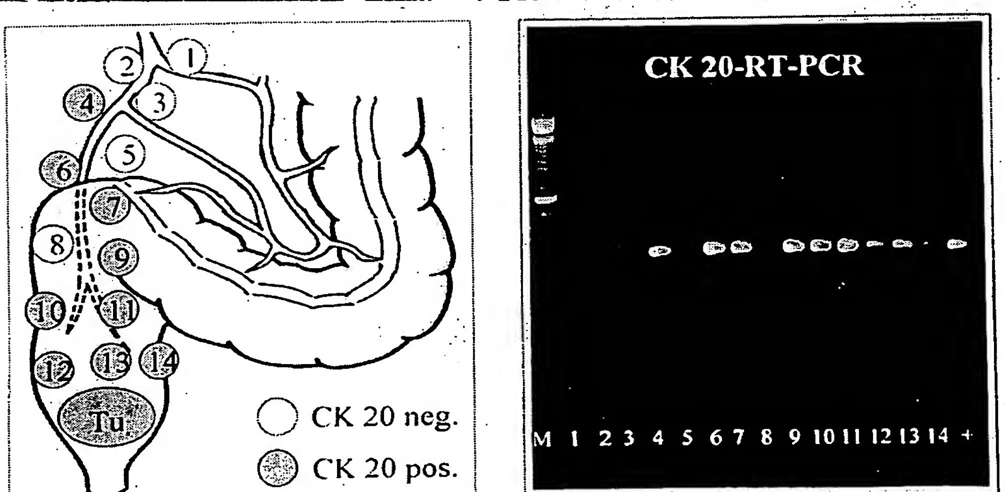

Fig. 1 Results from a patient with rectal carcinoma $pT_3 pN_0$. *Left*, surgical specimen with the location of tumor and lymph nodes; *right*, CK 20-RT-PCR amplification products of resected lymph nodes. *Tu*, tumor; *M*, molecular weight marker; +, positive control (tumor).

DISCUSSION

Although most patients with colorectal cancer are potentially curable by surgery at presentation, one-half of them will eventually die of the disease. This tumor progression could result from isolated disseminated tumor cells—in lymph nodes, blood, or bone marrow—that are not detected by current staging methods. The objective of adjuvant therapy is to eradicate viable disseminated tumor cells, thereby decreasing disease relapse and improving patient survival (31). Candidates for postoperative adjuvant therapy are patients at high risk for disease relapse, as judged by current clinical and pathological staging. In the group of patients without distant metastases, lymph node metastases are the most important prognostic factor (10). Consequently, adjuvant chemotherapy is recommended for patients with positive lymph nodes (UICC stage III; Ref. 32). For patients with colon cancer UICC stage I or II, adjuvant chemotherapy did not

Table 2 Results of the comparative analysis of lymph nodes, blood, and bone marrow samples in patients UICC stage I and II by CK 20-RT-PCR

Patient No	Stage	CK 20-RT-PCR	Location of CK 20-positive nodes			Blood	Bone marrow
			Central	Main vessel	Paracolonic		
1	I	Pos[a]	Pos	Pos	Pos	Neg	n.o.
2	II	Neg	Neg	Neg	Neg	Neg	Neg
3	I	Pos	Neg	Neg	Pos	Neg	n.o.
4	I	Pos	Pos	Pos	Pos	Neg	Neg
5	I	Pos	Neg	Pos	Pos	Pos	Pos
6	I	Pos	Neg	Neg	Pos	Neg	Neg
7	I	Neg	Neg	Neg	Neg	Neg	Pos
8	II	Pos	Neg	Pos	Pos	Neg	Neg
9	II	Pos	Neg	Pos	Pos	Neg	Neg
10	I	Pos	Neg	Pos	Pos	Neg	Neg
11	I	Pos	Neg	Pos	Pos	Neg	Neg
12	I	Pos	Pos	Pos	Pos	Neg	Neg
13	I	Pos	Pos	Pos	Pos	Neg	Neg
14	II	Pos	Pos	Pos	Pos	Neg	Pos
15	II	Pos	Pos	Pos	Pos	Neg	Neg
16	I	Pos	Neg	Pos	Pos	Pos	Neg
		14 of 16 87.5%	6 of 16 37.5%	12 of 16 75%	14 of 16 87.5%	2 of 16 12.5%	3 of 14 21.4%

[a] Pos, positive; Neg, negative; n.o., not obtained.

achieve a survival benefit, and, thus, adjuvant therapy is not recommended in these stages (32). Although considered at low risk, 30–40% of patients with colorectal cancer UICC stage I and II ultimately develop recurrent disease (1). It is in this patient group that prognostic markers may identify a patient subgroup at high risk for disease relapse who may also benefit from adjuvant therapy, especially because antitumor agents with a low systemic toxicity (for example, monoclonal antibodies) that have also been proven to be effective against dormant tumor cells are now available (33). Because tumor relapse is presumably caused by hematogenic or lymphogenic disseminated tumor cells, the detection of these disseminated tumor cells may identify patients who could benefit from adjuvant therapy.

Focusing on hematogenic tumor cell dissemination, Lindemann *et al.* (2), using immunocytological methods, detected disseminated colorectal cancer cells in bone marrow aspirates in 32% of patients. Tumor cell detection was clearly related to an earlier relapse and decreased survival of the respective patients. RT-PCR-based protocols have further improved the sensitivity and specificity of detection systems for disseminated cancer cells, allowing the identification of approximately one neoplastic cell in 10^7 normal peripheral mononuclear blood cells (3). CK 20 transcripts appear to be good targets for the detection of disseminated colorectal cancer cells because they are expressed in gastrointestinal epithelia, urothelium, or Merkel cells and in their respective tumors but not in other nontransformed tissues (4, 5). Burchill *et al.* (6) described a CK 20-RT-PCR system for the detection of malignant colonic epithelia in blood and bone marrow samples. Soeth *et al.* (7, 8) used a CK 20-RT-PCR assay to detect disseminated cancer cells in bone marrow aspirates and blood samples of gastrointestinal cancer patients. Using a modified CK 20-RT-PCR assay, we were able to detect circulating tumor cells in 24 of 58 patients with colorectal cancer in correlation to the tumor stage and in 6 of 7 patients who underwent hemihepatectomy for liver metastasis of colorectal cancer (9). We were also able to demonstrate significant intraoperative tumor-cell dissemination in these patients (9).

Because lymph node involvement has proven to be one of the most important prognostic factors for colon cancer patients (10), lymphogenic dissemination of tumor cells is likely to be at least as important as hematogenic cancer cell spread. The routine histopathological methods of lymph node examination show limited sensitivity because clinical pathologists investigate only one or two sections of each lymph node. In addition, the detection of small clusters of tumor cells is difficult. In about one-fifth of the cases primarily diagnosed as lymph-node negative, metastasis can be detected on reexamination by serial sectioning of the lymph nodes (11). Goldenberg *et al.* (12) introduced immunohistochemistry for the detection of tumor cells in lymph nodes that were previously considered free of disease by the standard H&E staining technique. Using immunohistochemistry, tumor cells were detected in about 3.6–6.1% of histopathologically negative lymph nodes (13–16). Of the reexamined patients, 25–36% had to be restaged from UICC stage I-II to UICC stage III. Using the combination of immunohistochemistry and the fat clearance technique, which allows the detection of more lymph nodes in a surgical specimen, even 55% of the reexamined patients had to be restaged upward (17). Because of the contradictory results in the published studies, however, the prognostic significance of immunohistochemically detected lymph node micrometastases remains controversial (13–16).

In an effort to further increase the sensitivity and specificity of tumor cell detection in lymph nodes, genetic methods

were applied. These methods have the advantage of a higher sensitivity and the possibility of examining not only single sections but multiple sections of a lymph node in a single examination. Using the mutant allele-specific amplification method, Hayashi *et al.* (18) were able to detect tumor cells of gastrointestinal carcinomas with mutant k-*ras* or *p53* genes in lymph nodes. However, this method is restricted to tumors with known k-*ras* or *p53* mutations. In addition, positive PCR results may originate from fragments of free tumor DNA rather than from viable tumor cells (19).

Mori *et al.* (20) and Liefers *et al.* (21) used CEA transcripts as targets for detection of micrometastasis in lymph nodes. However, the sensitivity of the used assay had to be reduced to avoid false-positive results. In the study performed by Liefers *et al.* (21), the detection of disseminated tumor cells was associated with an unfavorable prognosis, which suggests that microinvolvement of local lymph nodes is indeed associated with increased relapse rates. However, several arguments have been raised against this study: (*a*) only seven lymph nodes per patient were investigated; (*b*) too few negative control samples were included (34); and (*c*) the sensitivity of the CEA-RT-PCR assay has not been appropriately addressed (35). In addition, concerns about the specificity of the marker (CEA) used for the detection of the cancer cells have again been put forward (36) because CEA transcripts are also detectable in normal lymph nodes of patients who are not suffering from colorectal cancer (37).

Other investigators used RT-PCR amplification of CK 20 (22, 23), matrilysin (24), or CD 44 (25) mRNAs for the same purpose. Tumor cells were detected in lymph nodes of 16–57% of patients with histopathologically negative lymph nodes. Recently, Futamura *et al.* (26) reported the detection of micrometastases by the combination of CEA-RT-PCR and CK 20-RT-PCR in the lymph nodes of 13 of 13 examined colorectal cancer patients at UICC stage I or II. Because only 30–40% of patients with colorectal cancer UICC stage I and II develop recurrent disease, this high detection rate raises doubts concerning the prognostic relevance of these findings.

In this study, we investigated the rate of tumor cell dissemination in the lymph nodes, blood, and bone marrow of 20 patients with colorectal carcinomas using a CK 20-RT-PCR assay.

The sensitivity of the system was evaluated in previous studies (9) and reproducibly allowed the detection of approximately 10 tumor cells in 10 ml of blood. Specificity is a major concern in RT-PCR systems used for the detection of disseminated tumor cells (3). CEA-RT-PCR, for example, is known to yield false-positive results in normal lymph nodes (20, 21, 26). Specificity of the used CK 20-RT-PCR was ensured by the measures discussed previously (9). Lymph nodes of 22 healthy patients undergoing surgery for nonneoplastic colorectal disease consistently tested negative with the established CK 20-RT-PCR. These results demonstrate that normal lymph nodes do not contain CK 20-expressing cells. Even in patients with inflammatory bowel disease, nonneoplastic colon cells do not reach the first local lymph node station. Thus, false-positive results due to the migration of normal colon epithelia are unlikely. There is also concern that DNA-based methods for tumor cell detection may detect free tumor DNA rather than viable tumor cells (19), thereby reducing the specificity of the method. Because free mRNA is extremely unstable, tumor cells detected by RT-PCR are highly likely to be viable.

Isolated disseminated tumor cells were detected by CK 20-RT-PCR in lymph nodes in 14 of 16 patients UICC stage I and II. In previously published RT-PCR-studies, tumor cells were detected in lymph nodes of 16–57% of tested patients (20–25). Given the higher sensitivity of RT-PCR in relation to immunohistochemistry, these published detection rates are surprisingly low compared with the immunohistochemical data revealing positive lymph nodes in 25- 55% of patients. This could be due to the low sensitivity of the hitherto tested RT-PCR assays (1 tumor cell in 1 $\times$ 10^4 to 5 $\times$ 10^5 peripheral mononuclear cells) because single and nonnested PCR-systems were used (22–25). The higher detection rate in our study is presumably the result of the higher sensitivity (1 tumor cell in 10^7 peripheral mononuclear cells) of the nested CK 20-RT-PCR. Using a combination of a nested CK 20-RT-PCR and CEA-RT-PCR, Futamura *et al.* (26) recently reported tumor cell detection in lymph nodes of all of the 13 examined patients UICC stage I or II.

In our study, tumor cells were detected in the lymph nodes of 87.5% of patients UICC stage I and II, and in the study of Futamura *et al.* (26) they were detected in 100% of patients UICC stage I and II. These detection rates are much higher than the known tumor recurrence rate in these stages. Thus, the detection of tumor cells in surgically removed lymph nodes by CK 20-RT-PCR cannot be a prognostic marker *per se*. Because it is well known that the prognosis of patients with histopathologically tumor-infiltrated apical lymph nodes is worse than the prognosis of patients with negative apical lymph nodes (38–44), we stratified the results of the 16 patients UICC stage I and II according to lymph node position: (*a*) in 8 (50%) of the 16 patients, tumor cells were detected only in lymph nodes along the main vessel and paracolonic lymph nodes without infiltration of the apical lymph node; (*b*) in 2 (12.5%) of the 16 patients, tumor cells were detected only in paracolonic lymph nodes. Because all of these lymph nodes were surgically removed by lymphadenectomy, they may be of no further prognostic significance; (*c*) in 6 (37.5%) of the 16 patients, however, tumor cells were also detected in the apical lymph node. Because 30–40% of patients UICC stage I and II will develop recurrent disease, it could be hypothesized that the detection of tumor cells in the apical lymph node by CK 20-RT-PCR indicates tumor cell dissemination beyond the margin of surgical lymphadenectomy. These patients may have a worse prognosis and could benefit from adjuvant therapeutic regimes. This hypothesis remains to be confirmed by a follow-up of our patient cohort.

The evaluation of the relative importance of lymphogenic *versus* hematogenic tumor cell dissemination in colorectal cancer could provide important data for additional therapeutic strategies. For example, extended surgical lymphadenectomy could be useless in the presence of multiple tumor cells in blood and bone marrow. Clinical studies that address the relative importance of lymphogenic *versus* systemic tumor dissemination, however, are rare (27). We compared lymphogenic and hematogenic tumor cell dissemination in patients with colorectal cancer by examining lymph nodes, blood, and bone marrow from each patient. Tumor cells were only detected in 12.5% of blood and

21.4% of bone marrow samples from patients UICC stage I or II. Thus, lymphogenic tumor cell dissemination seems to be much more common than detectable hematogenic tumor cell spread in early colorectal cancer. These results are strong arguments for the importance of meticulous lymph node dissection, especially in patients with colorectal cancer in early tumor stages without systemic hematogenic micrometastasis. Adequate surgical lymphadenectomy may, therefore, be the most important factor to prevent recurrent disease in early colorectal cancer by resection of all of the potentially involved lymphatic tissue with the elimination of all of the lymphogenic disseminated tumor cells.

We conclude that the CK 20-RT-PCR is a sensitive and specific tool for the detection of lymphogenic and hematogenic dissemination of colorectal carcinoma. The long-term follow-up of our patients will provide data on the prognostic relevance of isolated disseminated tumor cells in the lymph nodes, blood, and bone marrow of patients with colorectal cancer and may lead to new concepts for adjuvant therapy.

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APPENDIX J

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olecular diagnosis of primary liver cancer by microsatellite DNA alysis in the serum

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Frequent loss of heterozygosity of microsatellites markers on specific chromosomal region have been reported in various types of primary human cancer. The same loss of heterozygosity has also been identified in the matched plasma/serum DNA. Using 109 microsatellite markers representing 24 chromosomal arms, we have examined the loss of heterozygosity in 21 cases of hepatocellular carcinoma, six of cholangiocarcinoma, and 27 cases of chronic hepatitis or cirrhosis. All cases of the hepatocellular carcinoma showed deletion from two to 10 chromosomal arms, while deletion of chromosomes from two to eight regions was detected in five of six cholangiocarcinoma patients. One or more loss of heterozygosity in the paired serum DNA could be detected in 16 of 25 (76.2%) hepatocellular carcinoma patients. In contrast, no alterations in serum DNA test could be found in cholangiocarcinoma patients. Five of seven (71.4%) hepatocellular carcinoma patients with alpha-fetoprotein levels less than 20 ng ml^{-1} produced positive serum DNA test. The profiles of 19 microsatellite markers gave a 100% positive predictive value and an 80.8% negative predictive value for hepatocellular carcinoma. In conclusion, we have determined a profile of microsatellite markers appropriate for differential diagnosis of primary liver cancer. The discovery may permit a high-throughput screening of hepatocellular carcinoma at an early stage of disease.
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Keywords: hepatocellular carcinoma; cholangiocarcinoma; microsatellite; loss of heterozygosity; molecular diagnosis

Genetics and Genomics

ıary liver cancer ranks fifth in frequency among all malignan- in the world with an estimated number of 437 000 new cases 990. The vast majority of primary liver cancer is hepatocellular inoma (HCC), a malignant tumour derived from hepatocytes. ℶ is increasing in many countries, particularly in areas where ıtitis C virus infection is more common than hepatitis B virus :tion (El-Serag and Mason, 1999). The cholangiocarcinoma), an intrahepatic malignant tumour which arises in the epithe- ı of the bile ducts, is the second common hepatic tumour. Its ırrence is largely high in areas where liver fluke infestation or ıhepatic stones is endemic, such as Hong Kong, Thailand Taiwan (Okuda *et al*, 1977).

he prognosis of HCC or CC is usually poor, with 3-year survi- rates estimated at 44.9% and 34% after hepatic resection, ectively (Okuda *et al*, 1977). In patients with symptomatic ℶ, the tumours are usually either too large and/or with local sion or distant metastasis. Therefore, only 10–20% of patients d be treated with potentially curative therapy, while the aining patients can only receive non-surgical therapy, such as anscatheter arterial embolisation or other local ablation treatments with a less satisfactory outcome. The patients with CC often present with obstructive jaundice at the stage of advanced stage. Currently, resection offers the only chance of cure and the best chance of long-term survival.

In order to improve the chances of successful treatment and patient survival, great effort has been devoted to detect HCC in the early stage when it is 'small', for example smaller than 3 cm, and where liver function reserve is good enough for curative therapy. Measurement of serum α-fetoprotein (AFP) and hepatic ultrasonography are widely used as surveillance modalities for high-risk patients. Elevated levels of AFP, with normal range set at 20 ng ml^{-1}, are detected in up to 75% of HCC patients, and values above 400 ng ml^{-1} are considered diagnostic for HCC (Ebara *et al*, 1986). However, 35% of HCC smaller than 3 cm may secrete little or no AFP into the circulation and thus will not be identified by this test (Ebara *et al*, 1986). As a result, measurement of AFP alone is not an ideal test for early diagnosis of HCC.

Recent advances in tumour genetics reveal that the genesis and progression of tumours follow an accumulation of multiple genetic alterations, including inactivation of tumour suppressor genes and/or activation of proto-oncogenes. Frequent loss of heterozygosity (LOH) of microsatellites markers on specific chromosomal region have been reported in various types of human cancer. In addition, nucleic acid-based molecular techniques have successfully identified the same LOH in the paired plasma/serum DNA from patients with diverse types of epithelial cancer, such as lung

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:hen *et al*, 1996; Sozzi *et al*, 1999, 2001), head and neck (Coulet al, 2000; Nunes *et al*, 2001), colon (Hibi *et al*, 1998), breast :hen *et al*, 1999; Silva *et al*, 1999), kidney (Goessl *et al*, 1998; isenberger *et al*, 1999). The results indicate that microsatellite NA analysis may have potential as a non-invasive test for cancer iagnosis. In this pilot study, we have searched for the genetic terations, using a total of 109 microsatellite markers representing ‡ chromosomal regions, in the serum DNA of patients with rimary liver cancer, i.e. HCC (n=21) and CC (n=6). The results iggest that microsatellite serum DNA analysis may be a valuable, on-invasive method for the early detection of primary liver incer.

IATERIALS AND METHODS

ample collection and DNA preparation

rimary tumour from 21 cases of HCC and six cases of CC ere collected from the Department of Surgery, National Cheng ung University Hospital, Tainan, Taiwan between September 998 and March 2000 (Tables 1 and 2). The tumour tissue as obtained immediately after surgical resection and stored : −80°C, and then microdissected as described in detail reviously (Eisenberger *et al*, 1999). We also enrolled 27 atients (age- and sex-matched) with a biopsy diagnosis of ironic hepatitis (n=20) or cirrhosis (n=7) as controls. Both eripheral blood lymphocytes and serum samples were obtained ith written informed consent. Briefly, DNA was extracted from eripheral blood lymphocytes, tumour and matched serum imples by digestion with 1% SDS/proteinase K (BD Biosciences lontech, CA, USA) overnight followed by phenol-chloroform traction and ethanol precipitation. Each tumour was reviewed ır histologic grading according to the Edmondson classification 1958). Clinical staging was determined according to the imour-node-metastasis staging protocol of the American Joint ommittee on Cancer (1997) with surveys of the clinical details, nage studies, and pathologic data. The clinical characteristics, ıcluding AFP, hepatitis B surface antigen (HBsAg) and hepatis C virus antibody (anti-HCV), were obtained from medical :cords.

Analysis for allelic loss

Microsatellite markers from selected chromosomal arms for PCR analysis were obtained from Research Genetics (Huntsville, AL, USA). The marker pairs selected for HCC (74 markers from 17 chromosomal arms) included 1p (D1S160, D1S163, D1S170, D1S186, MYCL), 3p (D3S1317), 4p (D4S394), 4q (D4S398, D4S395, D4S392, D4S422, FGA, FABP2, D4S427, D4S415, D4S1615, D4S1554, D4S1426, D4S1598, D4S620, D4S1566, D4S1545, D4S2920, D4S2943, D4S2954), 5q (D5S409), 6q (D6S264), 8p (D8S261, D8S277), 8q (D8S85, D8S200, D8S555, D8S283), 9p (D9S169, D9S1747, D9S104), 10q (D10S109), 11p (D11S554, D11S436, D11S1344, D11S932, D11S1324), 11q (D11S938, D11S29), 12p (D12S93), 13q (D13S171, D13S153, Rb1, D13S133, D13S227, D13S159, D13S166, D13S168), 14q (D14S72, D14S51), 16p (D16S419, D16S409, D16S3106, D16S498), 16q (D16S415, D16S408, D16S512, D16S289, D16S402, D16S516, D16S422, D16S413), and 17p (D17S520, D17S1176, TP53, D17S513, D17S578, D17S796, D17S849) based on previous reports (Yeh *et al*, 1996; Nagai *et al*, 1997; Piao *et al*, 1998; Fujii *et al*, 2000; Okabe *et al*, 2000; Yeh *et al*, 2001). The marker pairs selected for CC (55 markers from 16 chromosomal arms) included 2p (BAT-26), 3p (D3S3667, D3S1578, D3S3582, D3S3560, D3S1581, D3S3729, D3S1588, D3S3648), 4 (D4S415, D4S413), 5q (D5S323, D5S417), 6p (D6S263), 6q (D6S292), 7q (D7S495, D7S486), 9p (D9S747, D9S171), 11p (D11S907, D11S569), 14q (D14S1436), 16q (D16S3094, D16S511,

Table 2 Clinical characteristics of patients with cholangiocarcinoma

No.	Age/Sex	Size (cm)	Grade	Stage (AJCC)	Deleted chromosomes
22	62/F	1.2	III	III	4q, 14q
23	75/M	5.0	I	III	3p, 4q, 8p, 9p, 13q, 14q, 16q, 17p
24	60/M	3.5	I	III	No deletions
25	46/M	3.3	II	III	3p, 4q, 9p, 16p, 17p
26	53/M	3.0	III	III	8p, 9p, 16q, 17p
27	71/F	2.0	II	II	11p, 17p

'able 1 Clinical characteristics of patients with hepatocellular carcinoma

lo.	Age/Sex	Size (cm)	Grade	Stage (AJCC)	HBsAg/ anti-HCV	Deleted chromosomes	AFP (ng ml^{-1})	Positive markers in serum DNA
	40/M	3.6	II	III	+/−	3p, 4q, 8p, 16p, 16q, 17p, 18	3200	None detected
	55/F	2.5	I	II	+/−	1p, 3p, 9p, 13q, 17p	18[a]	D1S170
	62/F	4.2	II	II	+/−	1p, 3p, 8q, 9p, 14q, 16p, 16q, 17p	15.7[a]	D1S160, D14S51, D16S402, D16S422
	47/M	1.5	II	I	+/ND[b]	3p, 4q, 8q, 9p, 13q, 14q, 16q, 17p	230	D9S171, D16S289
	45/M	13.5	II	IV	+/−	1p, 4q, 5q, 8p, 8q, 9p, 13q, 14q, 16q, 17p	4450	D1S163, D5S409
	31/F	10.0	I	III	+/−	1p, 4q, 8p, 9p, 13q, 14q, 16p, 16q, 17p	44180	D9S747
	44/M	4.0	II	II	+/−	9p, 14q, 16q,	17190	D16S498
	48/M	14.0	IV	IV	−/−	1p, 8q, 13q, 14q, 16q	165000	D1S163, D8S555
	51/M	4.0	III	II	+/−	1p, 10q, 14q, 16q, 17p	6.9[a]	D1S163
	40/M	4.5	II	II	−/+	1p, 4q 10q, 13q, 14q	1.4[a]	D1S170
	64/M	3.5	III	II	−/+	1p, 4q, 8p, 13q, 16p, 16q, 17p	2560	D13S227
	66/M	5.0	II	II	−/+	1p, 3p, 4q, 5q, 8p, 8q, 11p	998.2	D1S170, D11S1344
	71/M	10.0	III	IV	+/−	1p, 4q, 5q, 8p, 9p, 13q, 14q, 16q, 17p	642.3	D9S171
	65/F	2.2	II	II	−/+	4q, 16q	127	D4S1545
	41/M	1.8	II	II	+/ND[b]	4q, 8p, 11p, 16q	435	None detected
	50/M	20.0	I	II	ND[b]	5q, 7q, 8q, 16p, 16q, 17p	3452	D5S409, D7S495, D8S200
	61/M	4.0	I	II	+/+	3p, 10q	5.8[a]	None detected
	44/M	14.0	II	II	+/ND[b]	1p, 8p, 16p	15.2[a]	None detected
	56/M	2.2	IV	III	−/+	4q, 8p, 9p, 13q, 17p	215.8	D4S1545
	60/M	2.5	I	II	ND[b]	1p, 4q, 5q, 8p, 13q	232	None detected
	65/F	1.3	I	I	−/+	9p, 16p, 16q, 17p	13[a]	D16S402, D16S516, D16S419

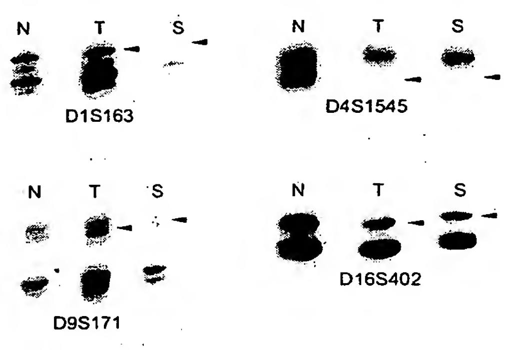
ᵃFP lower than 20 ng ml^{-1}. [b]ND=None detected.

;S534, D16S520), 17 (D17S695), 18q (D18S67, D18S51, ;S535), 20 (D20S85), 21q (D21S1245, D21S1436, D21S1270), Xp (DXS538) (Ding *et al*, 1993; Fujii *et al*, 2000). All of the ıours, irrespective of histopathology, were analysed for a total 09 microsatellite markers (74 markers for HCC and 35 markers CC) to confirm the specificity of chromosomal alterations as as their presence in the serum as tumour markers.

'rior to amplification, one primer from each pair was end- :lled with [γ-^{32}P]ATP (20 μCi; Amersham Pharmacia Biotech , NJ, USA) and T4 kinase (New England Biolabs Inc., MA, \) (Eisenberger *et al*, 1999). The amplification conditions for /merase chain reaction (PCR) were 1 × buffer (GibcoBRL, USA), 1.5 mM $MgCl_2$, 0.6 mM dNTP, 5 pmol of each primer, μl γ-^{32}P-labelled primer, 4 μl DNA, and 0.1 μl of Taq DNA /merase (GibcoBRL, NY, USA) in a total of 10 μl. PCR for each ner set was performed for 35 cycles consisting of denaturation 5°C for 30 s, annealing at 50–60°C for 1 min, and extension at C for 1 min (Perkin-Elmer Gene Amp PCR System 9600, kin-Elmer Corporation, MA, USA). The reaction products were ırated on 7% urea- formamide-polyacrylamide gels (GibcoBRL, USA) and exposed for 12–24 h. For informative tumours, H was scored if the tumour allele demonstrated a greater than ó reduction in intensity compared to the corresponding normal trol visually as previously described (Figure 1) (Eisenberger *et* 1999). Each LOH was confirmed by two independent experi- ıts. Allelic loss was determined by observation of LOH at any ırmative marker mapped to the same chromosomal region. n, LOH analysis was performed on matched serum samples.

tistical analysis

lic status was correlated with clinical and pathologic factors of C, such as histological grading, disease stage, tumour size, ım AFP levels, portal vein thrombosis and satellite nodule nation by Fisher's exact test. To evaluate the diagnostic accu- r in the microsatellite analysis, we considered four events: (A) itive test: presence of LOH in the primary tumour and in the ched serum DNA (true positive); (B) positive test: absence of H in tumour but presence of LOH in patient serum (false posi-); (C) negative test: presence of LOH in tumour but absence in ed serum samples (false negative); and (D) negative test: ence of LOH either in tumour or in paired serum samples (true ative). Decision diagnostic criteria were obtained from the ılts A, B, C, and D. Test sensitivity is equal to A/A+C. Test cificity is equal to D/D+B. Test accuracy is equal to: A+D/ 3+C+D. Predictive positive result or positivity prediction is al to A/A+B. Predictive negative result or negativity prediction qual to D/D+C.



ıre 1 LOH was scored for if the tumour allele (T) had a greater than reduction in intensity compared to the corresponding allele in the nor- control (N). Identical loss, highlighted by arrowhead, was also demon- ed in the matched serum (S).

RESULTS

All of the HCC cases showed deletion from two to 10 chromosomal arms (Table 1). The highest incidence was 16q (66.7%), followed by 1p (57.1%), 4q (57.1%), 8p (52.4%), 17p (52.4%), 13q (47.6%), 14q (42.9%), 9p (33.3%), 16p (33.3%), 3p (28.6%), 8q (28.6%), 5q (23.8%), 10q (14.3), 11p (9.5%), 18 (4.8%), and 7q (4.8%), respectively. The allelic losses were compared with biologic indicators of HCC, such as histological grading, tumour stage, size, and AFP serum levels (Table 1). A positive correlation was observed for 4q with AFP greater than 20 ng ml^{-1} (P=0.02), and 13q with satellite lesion (P=0.04) (data not shown). There was a trend toward positive association of 8p or 13q loss with advanced tumour staging (P=0.06), and 8p loss with AFP greater than 20 ng ml^{-1} (P=0.06). LOH on 14q and 16q occurred more frequently in hepatitis C virus-negative patients ($P<0.05$, respectively). Otherwise, patterns of allelic loss did not relate to histological grading or tumour size ($P>0.1$, respectively). In addition, novel alleles indicative of microsatellite instability were found in three patients (cases 2, 3 and 7, respectively) at chromosome 2, 13 and 16, respectively (data not shown).

As for tumour diagnosis, a total of 16 of 21 (76.2%) HCC patients were found to have LOH in serum DNA from one to four microsatellite markers (Table 1). The presence of LOH in the serum DNA did not relate to histological grading, staging or tumour size ($P>0.1$, respectively). If the cut-off values were set at AFP 400 ng ml^{-1}, negative serum LOH test was observed in two of 10 (20%) patients with abnormal AFP level (cases 1 and 15), and in three of 11 (27.3%) patients having AFP level within normal limits (case 17, 18 and 20). In contrast, five of seven (71.4%) patients (cases 2, 3, 9, 10 and 21) having AFP less than 20 ng ml^{-1} had positive LOH test. The results imply that microsatellite DNA analysis may have potential as a non-invasive diagnostic test for the early detection of HCC, especially for those who having AFP^{-}below reference range. However, none of three cases showing microsatellite instability in primary tumours had the same alterations detected in the serum DNA (data not shown).

With regard to cholangiocarcinoma, five of six patients belong to advanced stage of tumour (Table 2). Deletion of chromosome was detected from two to eight regions in five of six patients. But, no any alterations in serum DNA test could be found, despite of the fact that some common allelic losses were observed between HCC and CC. The results seem to imply a limited potential of the microsatellite analysis in diagnosing of CC.

Altogether, the data of this pilot study indicate that microsatellite analysis of serum DNA could successfully detect around three-quarters (76.2%) of HCC by screening the following profile of microsatellite markers, i.e. D1S160, D1S163, D1S170, D4S1545, D5S409, D7S495, D8S200, D8S555, D9S171, D9S747, D11S1344, D13S227, D14S51, D16S289, D16S402, D16S419, D16S422, D16S498, D16S516. The positive predictive value was 100%, and negative predictive value was 80.8%.

DISCUSSION

Currently, only ultrasound-guided needle biopsy yields diagnostic material and allows a definitive differential diagnosis between HCC and CC (Tsuji *et al*, 1999). Measurement of serum AFP and hepatic ultrasonography are widely used as surveillance modalities for patients with high-risk of liver cancer. In patients with HCC, however, around one-third of small tumour (<3 cm) was shown to secrete little or no AFP into the circulatory system (Ebara *et al*, 1986). As a result, it is mandatory to explore supplementary tumour marker for early detection of HCC.

In this study, we detected one or more LOH in the serum DNA in three-quarters of HCC patients. The results are comparable to some prior reports showing microsatellite alterations in the plas-

a/serum from cancer patients (Chen *et al*, 1996, 1999; Goessl *et* , 1998; Hibi *et al*, 1998; Eisenberger *et al*, 1999; Silva *et al*, ›99; Sozzi *et al*, 1999, 2001; Coulet *et al*, 2000; Nunes *et al*,)01). However, the sensitivity of microsatellite DNA test greatly :pends on the kind of cancer studied. For example, the detection te was 71% for small cell carcinoma of the lung (Chen *et al*, ›96), from 60 to 63% for renal cell carcinoma (Goessl *et al*, ›98; Eisenberger *et al*, 1999), between 48 and 66% for breast ncer (Chen *et al*, 1999; Silva *et al*, 1999), from 40 to 45% for ›n-small cell lung cancer (Sozzi *et al*, 1999, 2001), between less an 2 and 18.7% for head and neck cancer (Coulet *et al*, 2000; unes *et al*, 2001) using a set of primer pairs.

In contrast, the lack of tumour-specific LOH in the serum of CC itients appears to correspond to the observation reported for ›lon cancer (0%) (Hibi *et al*, 1998), though these patients may ill have very low levels of tumour-derived DNA. Nonetheless, e difference appears to support the propensity of HCC to spread vascular system (portal vein and hepatic vein), whereas peri-:ural invasion and lymphatic involvement are frequently present CC at an early stage (Sasaki *et al*, 2001). Taken together, the ita support the detection of circulating tumour-derived DNA as supplementary tumour marker in the differential diagnosis of :imary liver cancer.

Of particular interest, LOH in the serum DNA could be detected five of seven HCC patients with serum AFP less than) ng ml^{-1}, the normal range for the healthy adults. Previous udy found that approximately one third of small HCC (less than cm) may secrete little or no AFP into the circulation (Ebara *et al*, ›86). Our data imply that the limitation of low specificity of AFP diagnosing small HCC might be compensated by microsatellite ialysis on cell-free DNA in cancer patients. It will be especially ıluable in patients with poor liver function or ascites, in which further diagnostic investigation is prohibited. A prospective study is required to validate the importance of this molecular technique in the multimodality therapy for HCC.

We showed that the detection of HCC (71.4% sensitivity and 100% specificity) is feasible by screening the serum samples with a profile of 19 microsatellite markers. However, a recent study reported that around 50% of the cirrhotic nodules are monoclonal and already have chromosomal aberrations (Yeh *et al*, 2001). Whether the aberrations are also present in the serum DNA is currently unknown. Wang *et al* (2000) reported 87% of sensitivity in detection of p15/p16 methylation in the circulating DNA of HCC patients. Although the results seem to be better than we have in monitoring the profile of microsatellite markers, both the ability of differential diagnosis of HCC from CC and their value in patients having AFP below reference range were not examined. As a result, the impact of these observations in the development of HCC and/or cell type-specific genetic markers needs to be clarified in a large cohort of liver disease patients.

In conclusion, we have determined a profile of molecular markers appropriate for differential diagnosis of primary liver cancer in the serum. The discovery may permit a high-throughput screening of hepatocellular carcinoma at an early, and potentially resectable, stage of disease.

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APPENDIX K

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Molecular detection of tumour DNA in serum and peritoneal fluid from ovarian cancer patients

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Summary We have analysed DNA extracted from the serum and peritoneal fluid of 20 ovarian cancer patients for the presence of tumour-specific genetic alterations. The 20 patients included six with stage Ia disease. Using six polymorphic microsatellite loci we were able to detect novel alleles or loss of heterozygosity in 17/20 serum samples and 12/19 peritoneal fluid samples. Tumour-specific abnormalities were detected in the serum of all but one of the stage Ia cases. Half of the occurrences of loss of heterozygosity identified in primary tumour material were detectable in the serum samples. Novel alleles indicative of microsatellite instability were found in 3/6 patients with stage Ia disease but in only 1/14 of patients with more advanced disease. One of the eight patients in the control group displayed abnormalities in her serum DNA. The ease with which tumour-specific alterations were detected in serum and peritoneal samples from ovarian cancer patients, using a panel of only six polymorphic microsatellite markers on four chromosomes, suggests that molecular detection methods could prove useful in the staging, monitoring and screening of this disease.

Keywords: ovarian cancer; molecular detection; microsatellite instability; serum

Ovarian cancer is the leading cause of death from gynaecological malignancy in the UK, with over 4000 women dying annually. The high mortality is attributable to the lack of effective screening strategies and the fact that early-stage disease remains clinically silent. Most patients present with advanced disease, for which the long-term survival remains <30%. However, if early stage disease could be detected the 5-year survival could rise to 80–95% (Berek, 1994). Current ovarian cancer screening methods using serum tumour markers and ultrasound/Doppler techniques lack the specificity to achieve this goal at present.

Nucleic acid-based molecular detection methods have been used to demonstrate the presence of tumour cells in samples of stool (Sidransky et al, 1992), urine (Mao et al, 1996), sputum (Mao et al, 1994) and blood (Smith et al, 1991). In addition, the same approaches have confirmed the presence of free circulating tumour DNA in the serum of patients with epithelial tumours of the lung (Chen et al, 1996), head and neck (Nawroz et al, 1996) and colon (Hibi et al, 1998). The majority of these studies have demonstrated clear clonality between the genetic alterations observed in test samples and the corresponding primary tumour material. The types of alteration recorded have included specific gene mutations, loss of heterozygosity (LOH) and microsatellite instability (MI).

Only 5–10% of ovarian carcinomas appear to involve a familial predisposition. The two major syndromes involved are the familial breast and ovarian cancer syndrome, associated with inherited mutations in the BRCA1 gene, and the Lynch II syndrome (HNPCC), associated with inherited defects in the DNA mismatch repair system. Abnormalities associated with sporadic forms of the disease include mutations in k-*ras* and *p53* genes, and changes in expression of *HER-2* (Gallion et al, 1995). Allelotyping studies have identified common sites of LOH on chromosomes 5q, 6q, 11p, 13q, 14q, 15q, 18q, Xp (Cliby et al, 1993; Osborne and Leech, 1994), and particularly on chromosome 7q (Zenklusen et al, 1995), 9q (Schultz et al, 1995) and 17p (Phillips et al, 1993). The occurrence of novel alleles at microsatellite loci, due to either a replication error phenotype (RER$^+$) resulting from defective mismatch repair or general genetic instability, has been described in 17–37% of ovarian tumours (Fujita et al, 1995; King et al, 1995; Sood and Buller, 1996). However, MI may occur in up to 75% of stage I ovarian tumours, and 71% of uncommon histopathological types such as endometrioid or mixed serous and mucinous tumours (King et al, 1995).

We obtained serum and peritoneal fluid from 20 women with epithelial ovarian carcinoma, and used a panel of six polymorphic microsatellite markers to detect LOH or novel alleles in the DNA extracted from the samples. The same analysis was carried out on tumour tissue microdissected from paraffin sections of the primary tumour from each case. The aim of this study was to establish whether tumour-specific biomarkers could be detected in the serum and peritoneal fluid of ovarian cancer patients, particularly those with early-stage disease.

MATERIALS AND METHODS

Sample collection

Samples were collected from 20 women undergoing laparotomy for ovarian cancer, and eight women with benign or physiological cysts. None of the women were known to carry a familial

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able 1 Staging, pathology, cytology and genetic analysis of ovarian cancer patients

atient	Stage[a]	Histology type and differentiation	Cytology[b]	Tumour		Serum		Peritoneal fluid	
				LOH[c]	MI[d]	LOH	MI	LOH	MI
12	Ia	Endometrioid, Grade 1	–	1/6	–	2/6	–	0/6	–
00	Ia	Endometrioid and mucinous, Grade 2	–	1/5	–	0/5	–	0/5	–
775	Ia	Mucinous, Grade 2	–	0/1	5	0/4	1	0/4	1
224	Ia	Mucinous, Grade 2	–	0/3	2	0/3	2	0/3	2
475	Ia	Serous, Grade 2	–	3/4	–	2/4	–	0/4	–
65	Ia	Endometrioid, Grade 1	–	1/3	1	2/3	1	NA	–
05	IIc	Serous, Grade 3	+	1/5	–	1/5	–	1/4	–
88	IIIc	Serous, Grade 3	+	2/3	–	2/3	–	2/3	–
65	IIIc	Papillary serous, Grade 2	–	3/3	–	3/3	–	1/3	–
31	IIIc	Papillary serous, Grade 2	–	4/5	–	2/5	–	1/5	–
42	IIIc	Serous, Grade 3	–	5/5	1	2/5	–	0/5	–
85	IIIc	Papillary serous, Grade 3	–	3/4	–	1/4	–	1/4	–
77	IIIc	Endometrioid & serous, Grade 3	+	0/4	–	2/4	–	1/4	–
776	IIIc	Papillary serous, Grade 3	+	0/3	–	1/3	–	0/3	–
930	IIIc	Serous, Grade 3	–	5/5	–	3/5	–	5/5	–
992	IIIc	Papillary serous, Grade 3	–	2/4	–	2/4	–	1/4	–
042	IIIc	Endometrioid, Grade 3	+	2/5	–	0/5	–	0/5	–
944	IIIc	Serous, Grade 3	–	0/4	–	1/4	–	0/4	–
571	IIIc	Papillary serous, Grade 3	+	3/4	–	1/4	–	2/3	–
21	IV	Endometrioid, Grade 3	+	3/4	–	0/4	–	3/4	–
			Total	39/80	9	27/83	4	18/78	3

[a]FIGO staging of primary tumour; [b]presence of malignant cells in ascitic fluid or peritoneal washings; [c]proportion of informative (heterozygous) markers isplaying loss of heterozygosity (LOH); [d]microsatellite instability. NA, not available for molecular analysis.

ıredisposition for ovarian cancer, although one woman had a first-legree relative with breast cancer. The cancer cases included six tage Ia and 14 stage II–IV cases. In all six of the stage Ia cases otal abdominal hysterectomy, bilateral salpingo-oopherectomy, ımentectomy and peritoneal washings for cytology were taken, vhile ipsilateral pelvic node resection and para-aortic node ampling was carried out in three of the six. The pathological taging, serum CA125 and cytology results are shown in Table 1.

)NA extraction

Vormal DNA

'en millilitres of blood were taken into heparinized tubes, and 1 nl was used for extraction of white blood cell DNA using Nucleon Scotlab).

Serum DNA

'en millilitres of blood was taken, allowed to clot and then entrifuged at 1300 *g*. The serum supernatant was frozen at –80°C n aliquots and DNA was extracted from 400 µl using DNAzol Gibco BRL).

Peritoneal fluid DNA

\t laparotomy, 20 ml of ascitic fluid or pelvic peritoneal washings vere taken, of which 10 ml were sent for routine cytological nalysis, and the other 10 ml for molecular analysis. The latter amples were centrifuged at 1300 *g* to pellet any cells, which were vashed twice in phosphate-buffered saline (PBS). The cells were rozen at –80°C in tissue culture-freezing medium (2.75 ml)ulbecco's modified Eagle's medium, 1.25 ml fetal calf serum, ml dimethyl sulphoxide (DMSO)) in 1 ml aliquots. Cells were pun out of thawed aliquots and washed twice with 5 ml PBS to emove DMSO. Epithelial cells were then harvested by immuno-ıagnetic separation using Dynabeads coated with the epithelial specific antibody Ber EP4 (Dynal UK). DNA was extracted from the cells using Dynabeads DNA Direct (Dynal UK). A peritoneal fluid sample from patient 065 was unavailable for molecular analysis.

Tumour DNA

Areas of tumour tissue were microdissected from ten paraffin sections of primary tumours and DNA extracted using Nucleon (Scotlab). The precipitated DNA was resuspended in 50 µl of water.

Cyst wall DNA

For cases of benign and physiological cyst, areas corresponding to the cyst wall were microdissected and processed in the same way as the tumour DNA.

Microsatellite analysis

Polymerase chain reaction (PCR) amplification was carried out using oligonucleotide amplimers specific for six polymorphic DNA microsatellite loci:

DP1 (D5S346) on 5q (Spirio et al, 1993)
486 (D7S486) on 7q (Gyapay et al, 1994)
522 (D7S522) on 7q (Gyapay et al, 1994)
D11 (D11S904) on 11p (Gyapay et al, 1994)
p53V (intron1 in p53) on 17p (Cawkwell et al, 1994)
BRCA1 (D17S855) on 17q (Gao et al, 1995).

The chosen markers map to regions which commonly display LOH in ovarian carcinomas. One amplimer from each pair was fluorescently labelled using the dye-amidite method (Cawkwell et al, 1994).

The PCR reactions were set up in 25 µl of 1 × Super*Taq* Reaction Buffer (HT Biotechnology Ltd) containing 200 µM dNTPs, 12.5 pmol of each amplimer, 1.5–6.0 mM magnesium

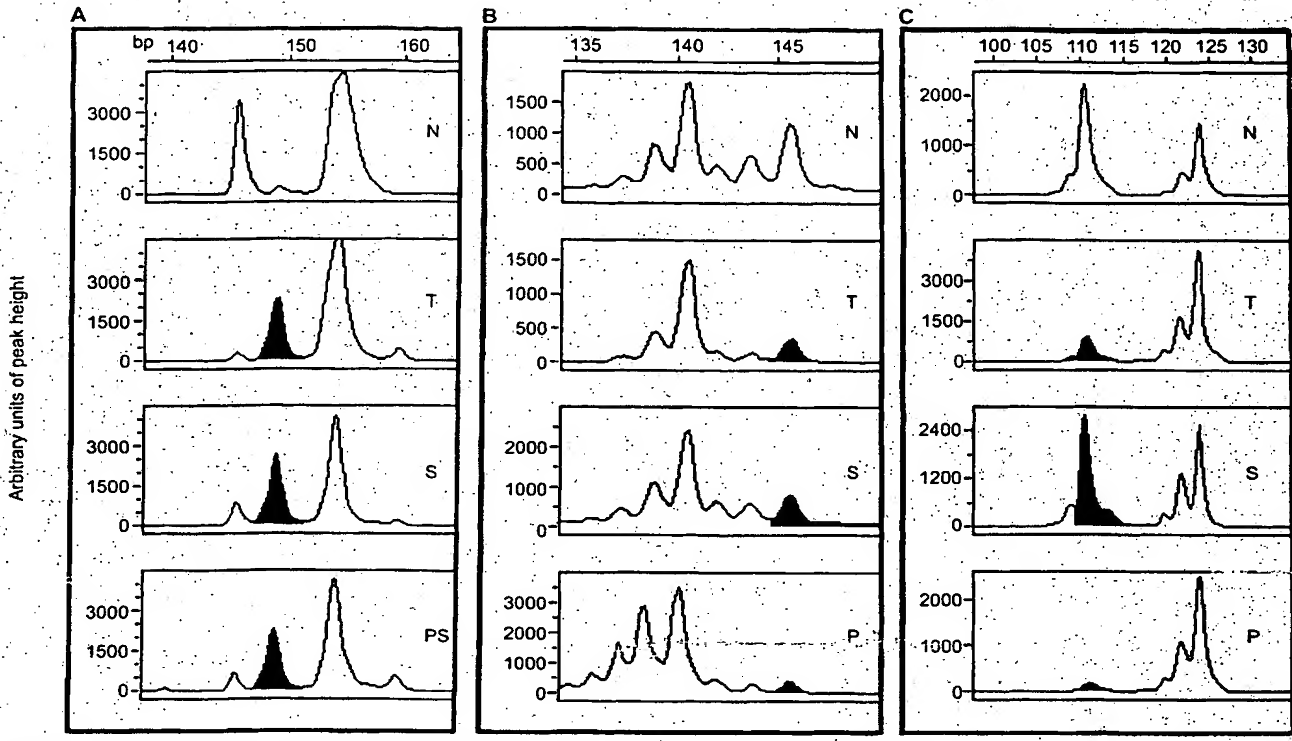

Figure 1 Electropherograms of microsatellite instability (MI) or loss of heterozygosity (LOH) in three ovarian cancer patients. Fluorescently labelled PCR products were run on an ABI 373A DNA Sequencer. The fluorescent signals were converted into electropherograms by Genescan Software. Length of product in base pairs is indicated on the X-axis and an arbitrary measurement of peak height, proportional to signal intensity, on the Y-axis. **(A)** MI at p53V locus seen in patient 4224; **(B)** LOH at the D7S486 locus seen in patient 165; **(C)** LOH at the D5S346 (DP1) locus seen in patient 4571. N, normal DNA; T, tumour DNA; S, serum DNA; P, peritoneal fluid DNA

chloride (optimized for each pair of amplimers), and 1 µl of DNA. After denaturing at 95°C for 3 min and a pause at 80°C to add 0.5 U of Super*Taq* polymerase (HT Biotechnology Ltd), the DNA was amplified using a programme of 92°C for 30 s, 56°C for 1 min, 72°C for 30 s, for a total of 35 cycles. The amplification products were visualized by agarose gel electrophoresis to estimate yield, electrophoresed on an ABI 373A DNA Sequencer (Applied Biosystems), and analysed using Genescan Analysis Software (Applied Biosystems), which determines the size of the PCR products and the amount of fluorescent signal. Suitable dilutions of PCR products were run to ensure that band intensities fell within the linear portion of the fluorescence detection range (500–4000).

Optimizing template DNA for PCR

We found that significantly different allele ratios could be obtained in our PCR reactions with different amounts of starting template DNA. It is difficult to accurately quantify the amount of DNA actually available for PCR amplification in a small sample. It was therefore essential to use different dilutions of normal control DNA (down to 1×10^{-4}) and choose the dilution which produced a comparable yield, as estimated by agarose gel electrophoresis, to that of the tumour/serum/peritoneal fluid samples as the control lane for Genescan analysis.

LOH and MI

The use of Genescan software and multiple PCR runs allowed us to accurately measure alterations in allele ratios. LOH was defined as a >20% shift in allele ratios (see below), as calculated using the equation $T_1{:}T_2/N_1{:}N_2$ (where $T_1{:}T_2$ is the ratio of the two tumour alleles and $N_1{:}N_2$ is the ratio of the corresponding normal alleles). Novel alleles were defined as one or more novel peaks consistent in size with expansion or contraction of the microsatellite repeat and distinct from PCR stutter products. All LOHs or MIs were confirmed by multiple independent PCR amplifications.

RESULTS

Defining parameters for LOH

An important consideration in studies which look for LOH in tumour DNA is deciding what constitutes a significant shift in allele ratio at heterozygous loci. The use of Genescan Analysis software allowed us to accurately compare allele ratios from tumour, peritoneal fluid and serum DNA samples, with those obtained from normal DNA. Analysis of over 250 of these comparisons revealed a clear bimodal distribution, with 99% of values falling between 0 and 80% or 85 and 100% (data not shown), with a striking gap between 80 and 85%. On this basis we assumed that an allele ratio shift of 0–15% represented normal variation in the amplification of alleles present in equimolar amounts in normal diploid DNA, whereas a shift of >20% represented a notable deviation from a balanced allele ratio. For the purposes of this study we therefore chose a >20% shift as the criteria for defining LOH in our samples. However, most allelotyping studies using microdissected paraffin-embedded tumour material tend to use a >50% shift to define LOH. We therefore also

ıble 2 Results of Genescan analysis of PCR products from 20 ovarian ıncer patients analysed for LOH or microsatellite instability at six icrosatellite loci

ıtient	Sample	DP1	522	486	D11	P53	BRCA1
2	Tumour	○	○	○	●	○	○
	Serum	○	○	○	●	●	○
	Per. fluid	○	○	○	○	○	○
)0	Tumour	○	○	○	○	×	●
	Serum	○	○	○	○	×	○
	Per. fluid	○	○	○	○	×	○
'75	Tumour	MI	MI	MI	MI	MI	○
	Serum	○	○	○	MI	×	○
	Per. fluid	○	○	○	MI	×	○
ː24	Tumour	○	×	○	MI	MI	○
	Serum	○	×	○	MI	MI	○
	Per. fluid	○	×	○	MI	MI	○
ı75	Tumour	●	×	○	●	●	×
	Serum	●	×	○	○	●	×
	Per. fluid	○	×	○	○	○	×
;5*	Tumour	●	MI	×	○	○	×
	Serum	●	MI	×	●	○	×
)5	Tumour	○	○	○	○	×	●
	Serum	○	○	●	○	×	○
	Per. fluid	NP	○	○	○	×	●
ı8	Tumour	×	×	○	●	×	●
	Serum	×	×	○	●	×	●
	Per. fluid	×	×	○	●	×	●
;5	Tumour	×	●	●	×	●	×
	Serum	×	●	●	×	●	×
	Per. fluid	×	○	●	×	○	×
ı1	Tumour	●	×	○	●	●	●
	Serum	●	×	○	○	○	●
	Per. fluid	○	×	○	○	○	●
ı2	Tumour	●	●	●	●	MI	●
	Serum	●	○	○	●	×	○
	Per. fluid	○	○	○	○	×	○
ı5	Tumour	○	×	●	●	×	●
	Serum	○	×	○	○	×	●
	Per. fluid	○	×	○	○	×	●
'7	Tumour	○	×	×	○	○	○
	Serum	○	×	×	●	○	●
	Per. fluid	○	×	×	●	○	○
'76	Tumour	○	×	○	○	×	×
	Serum	○	×	○	●	×	×
	Per. fluid	○	×	○	○	×	×
ı30	Tumour	●	●	●	●	×	●
	Serum	●	●	●	○	×	○
	Per. fluid	●	●	●	●	×	●
ı92	Tumour	●	×	×	●	○	○
	Serum	●	×	×	○	●	○
	Per. fluid	●	×	×	○	○	○
ı42	Tumour	●	○	○	○	×	●
	Serum	○	○	○	○	×	○
	Per. fluid	○	○	○	○	×	○
ı44	Tumour	×	○	○	○	×	○
	Serum	×	○	●	○	×	○
	Per. fluid	×	○	○	○	×	○
;71	Tumour	●	×	●	○	×	●
	Serum	●	×	○	○	×	○
	Per. fluid	●	×	○	NP	×	●
ː1	Tumour	●	○	×	●	×	●
	Serum	○	○	×	○	×	○
	Per. fluid	●	○	×	●	×	●

– heterozygosity retained; ● – loss of heterozygosity; MI – microsatellite stability; × – non-informative; NP – no product was obtained from repeated ːR amplifications. *No peritoneal fluid sample was available for molecular ıalysis from this patient. Per. fluid, peritoneal fluid

analysed our results using a >50% shift in allele ratio as the criteria for LOH (see below).

LOH and MI in primary tumour

All of the primary tumours displayed at least one genetic alteration (Figure 1A–C), with the exception of patients 577, 0776 and 3944 (Table 1). LOH was detected at 39/80 (49%) informative markers, and was twice as common in stage II–IV (33/58, 57%) as in stage Ia tumours (6/22; 27%). LOH occurred most frequently at BRCA1 on chromosome 17q (63%), DP1 on 5q (56%) and D11 on 11p (53%) (Table 2).

Novel alleles were observed in the primary tumours of 3/6 stage Ia patients (3775, 4244, 065), but only in 1/14 patients (042) with more advanced disease (Tables 1 and 2). In the case of patients 3775 (novel alleles at 5/6 loci) and 4224 (2/6) this may be evidence of an RER+ phenotype associated with mismatch repair deficiency.

LOH and MI in serum DNA

Genetic alterations were detected in 17/20 serum DNA samples, including 5/6 of the samples from patients with stage Ia disease (Table 1). LOH was harder to detect in serum (27/83 or 33% of markers) than in the primary tumours (49%). Of the 27 cases of LOH detected in serum, 19 (73%) were observed in the corresponding primary tumour (Figure 1B,C) and eight were specific to the serum DNA (Table 2). Thus 19/39 (49%) examples of LOH found in primary tumours were detectable in serum DNA.

Novel alleles were harder to detect in serum DNA than in primary tumour DNA (Table 1). Of the nine examples found in primary tumours, four were detected in the corresponding serum samples (Table 2 and Figure 1A). The three patients with novel alleles in their serum DNA all had stage Ia disease (Table 1).

LOH and MI in peritoneal fluid DNA

Only 12 of the 19 DNA samples extracted from peritoneal fluid exhibited genetic alterations, and only 18/78 (23%) heterozygous markers displayed LOH compared to 33% in the serum samples and 49% in the primary tumours (Table 1). However, 17 of the 18 examples of LOH in the peritoneal fluid samples were also found in the corresponding primary tumour (Figure 1B,C), indicating a higher degree of clonality than was seen with the serum samples (Table 2).

Novel alleles were detected in peritoneal fluid DNA from two of the stage Ia patients, all of which had been observed in the primary tumour and serum from the same patients (Table 2 and Figure 1A). By definition, these two patients had negative peritoneal fluid cytology.

Reanalysis of results using >50% in allele ratios

Out of 84 examples of LOH featured in Table 1, 60 displayed >50% shift in allele ratio. Of the remaining 24, which showed a 20–50% shift, 19 coincided with the finding of a >50% shift in another sample from the same patient. For example, the serum sample from patient 4571 showed a 30% reduction in the shorter allele at marker DP1 (Figure 1C), whereas the tumour and peritoneal fluid samples showed almost complete loss. These observations of significant ($P < 0.001$) clonal similarity support our initial

assumption that a >20% shift represents a genuine case of LOH. However, taking a >50% shift as the criteria for LOH we would still have detected tumour-specific alterations in 5/6 serum samples from stage Ia patients but only 7/14 stage II–IV patients. In the case of peritoneal fluid we would still have detected alterations in 2/5 stage Ia samples, and 8/14 stage II–IV samples (reduced from 10/14).

LOH and MI in control cases

DNA was extracted from serum, peritoneal fluid and paraffin-embedded cyst wall samples from four patients with benign mucinous cystadenoma of the ovary and four with physiological ovarian corpus luteal cysts, as controls to evaluate the level of false positive results using this approach. The three samples from each of eight patients were analysed for alterations at all six loci by comparison with normal DNA extracted from white blood cells. Out of a total matrix of 144 genetic analyses, only two displayed genetic abnormalities; an MI at p53 and an LOH at 522, in the serum of a patient with a physiological cyst (results not shown).

DISCUSSION

We were able to detect genetic alterations in the free circulating DNA extracted from the serum of 17/20 (85%) patients with ovarian carcinoma, using a panel of six microsatellite loci. In addition, exfoliated cells from 12/19 (63%) peritoneal fluid samples were found to contain tumour-specific alterations. For such a limited panel of genetic markers this represents a relatively high degree of sensitivity. Given that all tumour cell populations will contain genetic alterations of some description it should be possible to approach 100% sensitivity using an optimized panel of genetic markers.

Significantly, it was possible to detect tumour-specific genetic markers in serum samples from five out of six patients with stage Ia disease. This suggests that detectable amounts of DNA are released into the blood from tumours confined to one ovary. Previous studies have shown that tumour DNA can be detected in the serum of patients with advanced head and neck (Nawroz et al, 1996), lung (Chen et al, 1996) and colon cancer (Hibi et al, 1998). Our results clearly demonstrate that the earliest defined stage of ovarian carcinoma is detectable using this approach.

Surprisingly, one of the control patients with a physiological cyst had two genetic alterations in her serum DNA which were not present in her peritoneal fluid or ovarian tissue. Although there was nothing in this patient's clinical findings to suggest malignancy, it is feasible that she may have had an occult neoplasm. Genetic alterations of this type should be highly specific for tumour cell populations. If the possibility of PCR artefacts are eliminated, molecular detection techniques used for screening purposes should theoretically approach 100% specificity.

It is not possible to identify the precise biological or physical pathways through which DNA from tumour cells can enter the blood. Tumour cell death can occur through apoptosis, senescence, necrosis or immunosurveillance, any of which could theoretically result in tumour DNA entering the blood stream. The relative contribution of these processes will vary from tumour to tumour, depending on the phenotype of the cells. For example, it has been suggested that the MI/RER$^+$ genotype may make cells more immunogenic, due to accumulation of amino acid alterations in cell antigens, and therefore better targets for immunosurveillance (Bicknell et al, 1996). This could explain the improved prognosis for colorectal cancer patients with this genotype (Lothe et al, 1993; Lukish et al, 1998). In the case of stage Ia ovarian carcinomas, which are by definition confined to one ovary, DNA is probably released directly into the blood stream. With more advanced disease, exfoliated cells in the peritoneal cavity could provide another route of entry of DNA into the blood stream via the lymphatic system.

Our study demonstrates a strong degree of clonality between serum, exfoliated peritoneal cells and primary tumour DNA. Nineteen of 26 and 17/18 alterations seen in the serum and peritoneal fluid, respectively, were also seen in the corresponding primary tumour (Figure 1A–C). However, similar studies in head and neck, lung and colon tumours show almost exclusive clonality (Chen et al, 1996; Nawroz et al, 1996; Hibi et al, 1998). In our study we analysed DNA from a single primary tumour block, usually one showing a preponderance of tumour tissue. Our results would suggest that in 27% of cases the tumour DNA in the serum originates from a subpopulation of cells not significantly represented in the sample microdissected from the primary tumour paraffin block. Such discrepancies may result from tumour heterogeneity and limitations in primary tumour sampling. Another possibility is that clonal subpopulations of cells carrying specific changes may undergo preferential cell death and hence be relatively underrepresented in primary ovarian tumours but enriched for in serum DNA. Previous studies on clonality of ovarian tumours have not addressed genetic heterogeneity within primary tumours.

We find a higher frequency of novel alleles in patients with stage Ia ovarian tumours (3/6) than with stage II–IV (1/14) (Table 1). This is in agreement with an earlier study by King et al (1995) who reported that 75% of stage I tumours had MI compared to 11% of stage II–IV tumours. In addition, LOH is less apparent in patients with stage Ia tumours (27%) compared to stage II–IV (57%). This suggests that stage Ia ovarian tumours are more likely to have an RER$^+$ phenotype than more advanced tumours. Studies on colorectal carcinoma show that patients with RER$^+$ tumours have a better prognosis, because of reduced survival for cells with MI (Lothe et al, 1993; Lukish et al, 1998). In an analogous way the presence of DNA carrying novel alleles in serum may indicate higher levels of tumour cell death and signal a better prognosis for stage Ia ovarian cancer patients.

Two patients with stage Ia disease (3775 and 4224) could technically be upstaged to stage Ic as a result of tumour DNA being detected in their peritoneal fluid. These patients had been diagnosed as stage Ia on the basis of a negative peritoneal fluid cytology result and confinement of disease to one ovary histopathologically. Accurate staging has a significant bearing not only on prognosis, but also on the possible use of adjuvant chemotherapy in this patient subgroup. In the case of head and neck cancer, a prospective multicentre trial is currently underway in the USA to evaluate the efficacy of molecular staging and its impact on surgical practice (Caldas, 1997). To our knowledge, this is the first report of the possible molecular staging of ovarian cancer.

At present, population-based screening for epithelial ovarian cancer is not feasible due to the relatively low prevalence (15/100 000), the lack of specificity of available screening techniques, the absence of a definitive precursor lesion, and the inability to determine a lag time for the development of widespread disease. Advances in transvaginal sonography and colour Doppler imaging

ʼan Nagell et al, 1995), as well as the use of serial CA125 ıeasurements (Skates et al, 1995) and complimentary serum ıarkers such as OVX1 (Woolas et al, 1993), have improved early ımour detection. However, the efficacy of periodic screening of ʼen high-risk patients has not been clearly established.)roegemueller, 1994). As molecular detection of tumour DNA in :rum or peritoneal fluid seems possible even in stage Ia disease, ıis technique may hold considerable promise as a secondary :reening tool after initial tumour marker or ultrasound abnormal-ies have suggested ovarian pathology. In terms of disease moni-ıring it will be interesting to observe if and when tumour DNA in ıe serum of these patients disappears after treatment, and to what ‹tent its reappearance in serum predates clinical and biochemical ʼidence of recurrence.

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APPENDIX L

Survival of Tumor Cells in Stem Cell Preparations and Bone Marrow of Patients with High-Risk or Metastatic Breast Cancer after Receiving Dose-intensive or High-Dose Chemotherapy[1]

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ABSTRACT

Purpose: **We evaluated whether dose-intensive or high-dose chemotherapy can eliminate micrometastases in high-risk breast cancer patients.**

Experimental Design: **We monitored cytokeratin (CK)/17-1A positive cells in the bone marrow (BM) and peripheral blood stem cells (PBSC) and studied Her-2/neu serum levels of patients with locally advanced (n = 13; group 1) and metastatic breast cancer (n = 30; group 2) using immunomagnetic separation, immunocytochemistry, and ELISA.**

Results: **CK+ cells were found in the BM of 3 of 13 (23%) group 1 patients before but not after chemotherapy, resulting in an overall survival (OS) of 92% after a median follow-up of 33 months. Contamination of PBSC in 2 of 9 (22%) patients was not associated with decreased survival. In group 2 patients, the CK+ rate was 60% (18 of 30 patients) before and 40% (4 of 10 patients) after therapy with an OS rate of 43% after 29 months. PBSC samples were positive in 7 of 24 (29%) patients. CK+ BM and PBSC led to a rapid progress and short OS, whereas tumor cell-free BM and PBSC resulted in a mean OS of 30 months. The antigen 17-1A was detected on most CK+ cells in both patient groups before therapy, on all of CK+ PBSC, and on CK+ cells in group 2 patients after therapy. Increased Her-2/neu levels were found in group 2 patients before chemotherapy.**

Conclusion: **Micrometastatic cells are present in PBSC grafts and can survive even high-dose chemotherapy. The presence of immunotherapeutic target antigens supports the idea that a combined chemoimmunotherapy might be successful in eliminating minimal residual disease.**

INTRODUCTION

The usefulness of HD[3] chemotherapy with autologous stem cell support has been under evaluation in several treatment trials of patients with locally advanced or metastatic breast cancer, and the final results of ongoing trials have to be awaited (1–4). In most treatment regimens, PBSCs are mobilized by conventional-dose chemotherapy plus granulocyte colony-stimulating factors (5, 6). These new strategies for cancer therapy have evoked a need for the detection of tumor cell contamination of blood or BM missed by conventional tumor staging procedures, because a significant correlation between tumor cell detection in BM and poor prognosis factors as well as decreased disease-free and overall survival have already been described for breast cancer patients (7–15). It is also well known that most of these cells rest in the G_0 phase of the cell cycle (16), and it has been demonstrated recently (17) that micrometastatic breast cancer cells show a considerable heterogeneity in the expression of carcinoma-associated cell-surface molecules including Her-2/neu, CO17–1A, MUC-1, and Lewis^Y. In addition to BM studies, enhanced serum levels of Her-2/neu were found in some patients with Her-2/neu positive tumors, and this subset of patients had a worse prognosis than individuals not expressing Her-2/neu in the primary tumor (18–20). Taking into account the reduced efficacy of chemotherapeutic agents in nonproliferating cells and the considerable heterogeneity of neoplastic cells, these data suggest that a combined therapeutic approach, including immunotherapy and purging of PBSC, might improve the survival. This approach was supported in clinical trials targeting Her-2/neu or the 17-1A antigen in breast (21, 22) and colorectal cancer (23). Furthermore, purging of PBSC grafts from patients with breast cancer resulted in effective elimination of tumor cells (24, 25).

In contrast to investigations with BM, the actual risk of tumor cell contamination of PBSC collections and its clinical significance have not been extensively investigated. Whereas Sharp *et al.* (26) showed that tumor cell contamination of PBSC conferred a worse prognosis, Stadtmauer *et al.* (27) demon-

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[3] The abbreviations used are: HD, high-dose; BM, bone marrow; CK, cytokeratin; IMS, immunomagnetic separation; Mab, monoclonal antibody; MNC, mononuclear cell; OS, overall survival; PBSC, peripheral blood stem cell; IC, immunocytochemistry; G-CSF, granulocyte colony-stimulating factor.

strated that contamination of PBSC was not correlated with decreased survival.

At present, IC using CKs as epithelial marker proteins is the most common method for tumor cell detection with a sensitivity of one tumor cell among 10^6 marrow cells (28). Furthermore, IMS techniques have been developed for enrichment of epithelial cells from larger samples of mononuclear cells with subsequent detection of tumor cells by IC (29–32).

In this study, we evaluated whether dose-intensive or HD chemotherapy can eliminate micrometastases in patients with locally advanced or metastatic breast cancer. For that purpose, BM and PBSCs were studied for CK+/17-1A+ cells at primary diagnosis during and after therapy using IMS followed by IC. In addition, it was elucidated whether the measurement of the extracellular domain of Her-2/neu in serum samples is able to detect minimal residual disease before and after chemotherapy.

PATIENTS AND METHODS

Patients and Treatment

Between June 1997 and June 1999, we studied 43 patients with locally advanced (n = 13) and metastatic breast cancer (n = 30) who were referred to the Department of Internal Medicine (Cancer Research), University Hospital of Essen. Patient characteristics at the time of entry into the study are summarized in Table 1 and Table 2. All of the patients gave written informed consent for the investigations, including the BM aspirations.

Pretreatment Staging

Staging procedures included computed tomography of head, chest, abdomen, and pelvis, radionuclide bone scan, and iliac crest bone biopsy (optional) plus BM aspiration and tumor marker evaluation. Additional radiological tests or biopsies of suspicious lesions were done individually according to the findings of the staging examinations.

Mobilization Chemotherapy

The patients described in this report were treated according to three different protocols for high-risk (group 1) and metastatic breast cancer (group 2), implying three different mobilization regimens. Patients treated in an adjuvant setting and those with resectable lesions not pretreated with anthracyclines received three cycles of epirubicin (45 mg/m^2; day 1 and 2) and cyclophosphamide (600 mg/m^2; day 1 and 2) plus G-CSF (filgrastim at 5 μg/kg of body weight or lenograstim at 150 μg/m^2) starting on day 5 until recovery of WBCs or until completion of stem cell collection. Treatment was repeated every 2 weeks, and PBSCs were collected after the second course of chemotherapy. Patients with metastatic disease who were pretreated with anthracyclines in an adjuvant setting received three courses of Taxol (175 mg/m^2) and cisplatin (50 mg/m^2) plus G-CSF every 2 weeks. Subsequently, cyclophosphamide (2 g/m^2) and G-CSF were given for stem cell mobilization. After stem cell collection, those patients with oligotopic recurrence were restaged and were assigned for either surgery or irradiation of the involved lesions. Within 24 h of surgery or in parallel with radiotherapy, folinic acid (500 mg/m^2 for 2 h) and 5-fluorouracil (2 g/m^2 for 24 h) were applied on a weekly-times-six schedule before the patients received HD chemotherapy.

Table 1 Adjuvant therapy: characteristics of group 1 patients

Characteristics	No. of patients
Total	13
Age [mean (range)]	48 (46–54)
Clinical staging	
II	5
III A	4
III B	4
Grading	
G_2	8
G_3	4
n.d.[a]	1
Histological type	
Infiltrating ductal	8
Infiltrating lobular	4
n.d.	1
No. of positive axillary lymph nodes	
Mean	17/24
Range	6/9–29/38
Estrogen/progesterone receptor status	
ER+/PR+	7
ER−/PR−	1
ER+/PR−	4
ER−/PR+	1
Menopausal status	
Pre	4
Peri	4
Post	5
Type of initial surgery	
Mastectomy	10
Breast-conserving surgery	3
Treatment	
High dose	8
Overall survival rate	8/8
Relapse	4/8
Standard therapy	5
Overall survival rate	4/5
Relapse	1/5

[a] n.d., not determined.

Patients with advanced recurrent disease received three courses of doxorubicin (50 mg/m^2) and docetaxel (75 mg/m^2) plus G-CSF starting on day 3. Stem cells were collected after the second and, if necessary, third cycle of doxorubicin/docetaxel (German Breast Cancer Dose Intensity Study (GEBDIS) protocol; Table 2).

Stem Cell Collection and Flow Cytometry

PBSC collection was performed with a Spectra blood cell separator (COBE BCT, Lakewood, CO). Flow cytometry analysis was done on a Coulter Epics XL (Coulter Corporation, Hialeah, FL). Details of the procedures have been described elsewhere (33).

Cryopreservation and Thawing of PBSC

At the end of each stem cell apheresis, the collected cells were aliquoted to provide an equal amount of CD34+ cells for each course of HD chemotherapy, and the volume was brought to 44 ml/bag either by centrifugation and removal of excess plasma or by adding PBS without Ca^{2+} and Mg^{2+} (Department of Pharmacy, University of Essen). The minimum number of

Table 2 Metastatic disease: characteristics of group 2 patients

Characteristics	No. of patients
Total	30
Age [mean (range)]	43 (26–57)
Clinical staging at first diagnosis	
I	6
II	11
III	5
III B	1
IV	4
n.d.[a]	3
Grading	
G_1	2
G_2	18
G_3	8
n.d.	2
Histological type	
Infiltrating ductal	26
Infiltrating lobular	4
Site of metastasis	
Bone	12
Lung	8
Liver	10
Lymph nodes	13
Other (skin/ovary)	5
No. of metastatic sites/patient	
1	1
2	3
3	2
>3	24
Estrogen/progesterone receptor status	
ER+/PR+	19
ER−/PR−	9
ER+/PR−	1
ER−/PR+	1
Menopausal status	
Pre	19
Peri	4
Post	7
Type of initial surgery	
Mastectomy	16
Breast conserving surgery	13
n.a.	1
Treatment	
GEBDIS Protocol	17
Overall survival rate	8/17
Complete remission	1
Partial response	7
No change	3
Progressive disease	6
Metastatic resectable disease	13
Overall survival rate	7/13
Complete remission	6
Partial response	1
No change	2
Progressive disease	2

[a] n.d., not detected; n.a., not applicable.

CD34+ cells/kg body weight and course of HD chemotherapy was set to 1.5 to 2.0 × 10^6 with an additional back-up bag that was infused after the last course of HD chemotherapy. The procedure has been described in detail before (33).

Thawing in a 37°C water bath and transfusion of the PBSCs were done at the patients' bedside after i.v. premedication with antihistamines, cimetidine, and prednisone. Cells were transfused by i.v. push with a 50-ml syringe after taking samples for sterility tests.

HD Chemotherapy

All of the patients received two courses of HD chemotherapy. For those treated in the adjuvant protocol or for resectable disease, the first treatment consisted of cyclophosphamide (2 g/m^2 for 1 h; day 1 to 3) and carboplatin (500 mg/m^2 for 1 h; day 1 to 3). The second course consisted of thiotepa (200 mg/m^2 for 1 h; day 1 to 3) and mitoxantrone (20 mg/m^2 for 1 h; day 1 to 3). Stem cells were infused on day 5 of each course, and G-CSF (filgrastim at 5 μg/kg of body weight or lenograstim at 150 μg/m^2) was started the same day until recovery of WBCs.

Patients with unresectable advanced disease received at first etoposide (500 mg/m^2 for 4 h; day 1 to 3), ifosfamide (4 g/m^2 for 18 h; day 1 to 3), and carboplatin (500 mg/m^2 for 18 h; day 1 to 3), and then thiotepa (200 mg/m^2 for 24 h; day 1 to 4) and cyclophosphamide (1500 mg/m^2 for 24 h; day 1 to 4). Stem cells were reinfused on day 5 and 7, respectively, and G-CSF was started the same day until recovery of WBCs (GEBDIS protocol; Table 2).

Treatment after HD Chemotherapy

Radiation Therapy. Patients with stage III breast cancer received radiotherapy after completion of HD chemotherapy. External beam radiation consisted of 50 Gy to the chest wall and supraclavicular and infraclavicular lymph nodes. Parasternal lymph nodes were irradiated with 50 Gy in case of central or medial tumor localization. Patients undergoing lumpectomy received a boost to the primary breast site depending on the extension of the surgery and the results (tumor-free sites) of the pathological examination. All of the other patients received radiation therapy individually depending on the sites of involvement.

Hormonal Therapy. Premenopausal patients with positive hormonal receptors who were treated in an adjuvant setting received luteinizing hormone-releasing hormone agonists for 2 years. Postmenopausal patients (based on patients' history or follicle-stimulating hormone serum level > 10 units/liter) received tamoxifen for 5 years.

Patients with advanced recurrent disease received tamoxifen for 5 years or until progression irrespective of their receptor status. Those who had been pretreated with hormonal therapy were switched to a different hormonal treatment. All of the other patients were treated individually based on the hospital oncologists' decision.

Sample Preparation. BM (10 ml) was aspirated under local anesthesia from the upper iliac crest from each patient by needle aspiration under the conditions of normal coagulation parameters. MNCs were isolated from heparinized BM (5000 units/ml bone marrow) by Ficoll-Hypaque density gradient centrifugation (density, 1.077 g/mol; Pharmacia, Freiburg, Germany) at 400 × *g* for 30 min. Interface cells were washed (400 × *g* for 15 min) and resuspended in PBS. Using a Hettich centrifuge (Tuttlingen, Germany), 2 × 10^6 cells (1 × 10^6/slide) were directly spun onto glass slides (400 × *g* for 5 min) coated with poly-L-lysine (Sigma Chemical Co., Deisenhofen, Germany).

Positive IMS. If enough MNCs were available, epithelial cells were isolated from 1 × 10^7 to 3 × 10^7 MNCs using Dynabeads Anti-Epithelial Cell uniform, magnetizable polystyrene beads (Dynal, Oslo, Norway), coated with a mouse IgG1 monoclonal antibody (Mab Ber-EP4) specific for two (M_r 34,000 and 39,000) glycopeptide membrane antigens expressed

on most normal and neoplastic human epithelial tissues, identical with the tumor-associated 17-1A antigen.

Samples of 1×10^7 to 3×10^7 MNCs or PBSCs were resuspended in 1 ml of separation medium, containing PBS with 1% BSA. Prewashed dynabeads (10×10^6; bead to target cell ratio, 4:1) were added to the cell suspension and incubated at 2–4°C on an apparatus that provides both gentle tilting and rotation for 30 min. Subsequently, the bead/cell suspension was placed on a magnet for 2 min. After discarding the supernatant, the rosetted cells were isolated by removing the vial from the magnet and resuspended in 2 ml of PBS/BSA. Cytospins were performed as described under sample preparation.

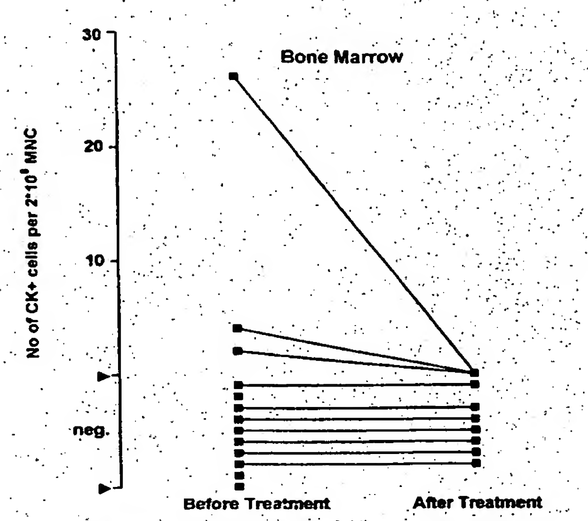
IC. After overnight air drying, staining for CK+ cells was performed as already described (17) using the Epimet kit (Micromet, Munich, Germany). The identification of epithelial cells by using this kit is based on the reactivity of the murine Mab A45-B/B3, directed against a common epitope of CK polypeptides. The kit uses Fab fragments of the pan-Mab complexed with alkaline phosphatase molecules. Briefly, the method includes: (*a*) permeabilization of the cells with a detergent (5 min); (*b*) fixation with a formaldehyde-based solution (10 min); (*c*) binding of the conjugate Mab A45-B/B3-alkaline phosphatase to cytoskeletal cytokeratins (45 min); and (*d*) formation of an insoluble red reaction product at the site of binding of the specific conjugate (15 min). Subsequently, the cells were counterstained with Mayer's hematoxylin for 1 min and finally mounted with aqueous permanent mounting medium containing 15 mM NaN_3 (Dako, Hamburg, Germany). A negative control antibody (conjugate of Fab-fragment; Micromet, Munich, Germany) served as a negative control. For each test, a positive control slide with the breast carcinoma cell line MCF-7 (American Type Culture Collection, Rockville, MD) was treated under the same conditions. The microscopic evaluation was carried out independently by two investigators.

Her-2/neu was determined using the Her-2/neu (c-erbB-2) sandwich enzyme immunoassay (Oncogene Science, Cambridge, MA), which uses a mouse Mab for capture and a different biotinylated mouse Mab for the detection of human neu protein. Both capture and detector reagents specifically bind to the extracellular domain of neu protein. The capture antibody has been immobilized on the interior surface of microplate wells. To perform the test, serum (dilution, 1:50 in dilution buffer), controls, and standards were incubated in the coated wells for 3 h at 37°C to allow binding of the antigen by the capture antibody. After a wash cycle, the immobilized antigen was mixed with the detector antiserum for 1 h at 37°C. After a second washing cycle, the amount of detector antibody bound to antigen was measured by binding with a streptavidin/horseradish peroxidase conjugate, which catalyzed the conversion of the chromogenic substrate *o*-phenylenediamine into a colored product. This colored product was quantitated by spectrophotometry and related to the amount of *neu* protein in the sample.

Evaluation of Data. Patients were evaluated as tumor cell positive if at least one CK+ cell was detected as analyzed by IC.

RESULTS

We studied CK+/17-1A+ cells in the BM and PBSCs of 43 patients with locally advanced (n = 13; group 1) or metastatic breast cancer (n = 30; group 2). The patient characteristics and clinical outcome are evaluated in Tables 1 and 2.



Serum [mean (range)]	Before Treatment	After Treatment
Her-2/neu (NR: -15ng/ml)	11 (6.1-19.94)	10.3 (9.4-12.8)

Fig. 1 Monitoring of CK+ cells in group 1 patients before and after treatment. ■, the number of CK+ cells/2×10^6 MNCs. The table under the figure summarizes the results for Her-2/neu before and after treatment; neg, negative; NR, normal range.

The results for micrometastatic cells in the BM and in PBSCs in group 1 patients before and after chemotherapy are shown in Fig. 1 and Table 3. With a median follow-up of 33 months, the OS rate was 92% with relapse free survival of 91%. Immunocytochemical BM involvement before chemotherapy could be demonstrated in 3 of 13 (23%) patients. A complete follow-up could be obtained in 9 of 13 patients with no micrometastatic cells found after chemotherapy in any patient analyzed.

Table 3 shows the detailed results for tumor cell detection in each group 1 patient. PBSC grafts were found to be positive in 2 of 9 (22%) patients. Interestingly, patient 3 showed a high frequency of CK+/17-1A+ cells in PBSCs and in the BM before chemotherapy but no CK+ cells after therapy. Furthermore, patient 12 had a high number of circulating CK+/17-1A+ cells in PBSCs but was negative for these cells in the BM before chemotherapy. None of these patients, including patients 8 and 9 with CK+ cells in the BM before chemotherapy, had a relapse up to now. A recurrence was only demonstrated for patient 2 after 12 months and patient 11 after 6 months, respectively. The epithelial cell surface antigen 17-1A was found on CK+ cells in 2 of 3 patients before chemotherapy and in all of the patients with CK+ PBSC grafts. Interestingly, enhanced Her-2/neu levels could only be demonstrated for patient 7 before chemotherapy.

The evaluation of micrometastatic cells and the results for soluble Her-2/neu for patients with metastatic disease are summarized in Fig. 2 and Table 4. With a median follow-up of 29 months, the OS rate was 43% with relapse free survival of 15%. At the time of diagnosis, 18 of 30 (60%) patients were found to

Table 3 Adjuvant therapy: monitoring of group 1 patients

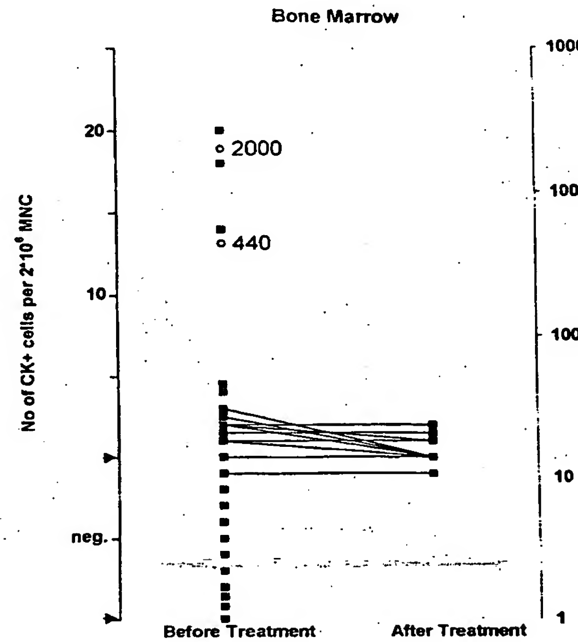
	CK+ cells/ 2 × 10⁶ MNC BM[a]	Her-2/neu	CK+ cells/ ml PBSC	CK+ cells/ 2 × 10⁶ MNC BM[b]	TTP[c] (mo)	OS (mo)
1	0		n.d.	0	36+	42+
2	0		0	n.d.	12	42+
3	26#		105#	0	31+	39+
4	0		0	0	23+	35+
5	0		0	0	26+	33+
6	0		0	0	17+	34+
7	0	Positive	0	0	25+	32+
8	2#		0	0	19+	29+
9	4		0	0	22+	28+
10	0		n.d.	n.d.	17+	25+
11	0		n.d.	0	6	15
12	0		110#	n.d.	26+	32+
13	0		n.d.	n.d.	LFU	LFU

[a] Before therapy.
[b] After therapy.
[c] TTP, time to progression; #, and 17-1A positive; LFU, lost to follow-up; n.d., not determined.

have CK+ cells in the BM. A complete follow-up was obtained in 10 of 30 patients with 4 of 10 (40%) patients remaining CK+ after chemotherapy. Markedly high levels were evaluated for Her-2/neu before chemotherapy.

The detailed data for patients with metastatic disease are shown in Table 4 and are divided into data for patients with complete follow-up and patients with incomplete follow-up. Evaluation was performed as indicated for patients with locally advanced breast cancer. In total, tumor cell contamination of PBSCs by immunocytochemical staining was found in 7 of 24 (29%) patients and correlated with decreased OS in 5 of 7 patients. The 17-1A antigen was present on CK+ cells of 12 of 18 (66%) patients before therapy (not determined in six cases), on all of the CK+ PBSCs, and on 2 of 4 CK+ cells after therapy. Although the number of completely monitored patients before and after chemotherapy was quite small, it is obvious that those patients (patients 5–7) with tumor cell contamination of BM and/or the PBSCs as well as enhanced serum Her-2 levels only had a partial response or no change to chemotherapy. On the other hand, those patients with no or a low number of micrometastatic cells in the BM before and after chemotherapy and no contamination of PBSCs (patients 1–4) could achieve a complete remission. Twenty of 30 patients were not completely analyzed for micrometastatic cells in the BM because of progressive disease, no application of a second course of HD chemotherapy, or refusal of marrow aspiration. As apparent from patients 14, 26, and 28–30, markedly enhanced numbers of CK+ cells in the BM and in PBSCs were associated with a rapid progress and short OS. On the other hand, tumor cell-free BM and PBSCs, as well as low Her-2 serum values, resulted in a mean overall survival of 30 months (patients 11, 16–18, and 23).

Serum analysis in this study group demonstrated enhanced levels of Her-2/neu in 6 of 30 patients (20%), resulting in a partial response or progressive disease and a short overall survival in most of the patients.



Serum [mean (range)]		
Her-2/neu (NR: -15ng/ml)	23 (8.9-45)	12.6 (9.9-16.6)
	Before Treatment	After Treatment

Fig. 2 Monitoring of CK+ cells in group 2 patients before and after treatment. ■, the number of CK+ cells/2 × 10⁶ MNCs. O values refer to the second scale (1–10,000). The table under the figure summarizes the results for Her-2/neu before and after treatment; neg, negative; NR, normal range.

DISCUSSION

Various immunocytochemical assays for epithelial tumor cells, including our own, have been shown to detect as few as one tumor cell among 1 × 10⁶ hematopoietic cells. IC using CKs, abundantly expressed as stable proteins in the majority of epithelial tumors, is currently the standard method for early detection of occult tumor cells in patients with solid tumors (28). Although it is still unclear whether this level of sensitivity is adequate, enrichment techniques can increase the sensitivity of tumor cell detection (29, 31, 32).

We combined IMS with IC and detected CK+ cells in 23% of group 1 patients and in 60% of group 2 patients before chemotherapy. Whereas no CK+ cells were found in patients of group 1 after chemotherapy, 4 of 10 (40%) patients of group 2 remained CK+ and 4 other patients who were positive before chemotherapy had negative aspirates afterward. After a median follow-up of 33 and 29 months, the OS rate was 92% in group 1 patients and 43% in group 2 patients, respectively. Increased numbers of CK+ cells in the BM and in PBSC collections were associated with a rapid progress and short overall survival, whereas patients with tumor cell-free BM and PBSCs, as well as low Her-2/neu serum values, had a mean overall survival of 30 months.

The fact that no CK+ cells could be detected in group 1 patients and in some group 2 patients after therapy are contradictory to the results by Braun *et al.* (14). Our findings might have different reasons. On the one hand, only unilateral BM

Table 4 Metastatic disease: monitoring of group 2 patients

	CK+ cells/2 × 10[6] MNC BM[a]	Her-2/neu	CK+ cells/ml PBSC	CK+ cells/2 × 10[6] MNC BM[b]	Response [TTP[c] (mo)]	OS (mo)
Complete follow up						
1	2		0	0	CR (4)	28+
2	2~		0	0	CR (12)	28+
3	1#		0	0	CR (11)	37+
4	1#		0	1#	CR (14)	31
5	2	Positive	6#	1	PR (11)	33+
6	1#		95#	1#	PR (14)	38+
7	0	Positive	5#	0	NC (10)	24
8	0		0	0	P (6)	36+
9	2~		0	0	P (8)	38
10	1~		0	1	P (21)	27
Incomplete follow up						
11	0		0		CR (20+)	22+
12	0	Positive	0		CR (14)	20
13	14#		0		CR (8)	LFU
14	4#		16#		CR (12)	17
15	4#		n.d.		PR (15)	32
16	0		0		PR (15)	34+
17	0		0		PR (11)	29+
18	0		0		PR (22+)	33+
19	18#	Positive	0		PR (10)	22+
20	0		n.d.		PR (2)	20
21	0		n.d.		NC	29
22	1#		n.d.		NC (2)	12
23	0		n.d.		NC (10)	35+
24	0		0		P	12
25	2#		0		P	6
26	440#	Positive	1828#		P	3
27	0	Positive	n.d.		P	3
28	2		60#		P	7
29	20#		0		P	8
30	2000#		16#		P	10+

[a] Before therapy.
[b] After therapy.
[c] TTP, time to progression; #, and 17-1A positive; ~, expression of the antigen 17-1A was not analyzed; CR, complete remission; LFU, lost to follow up; NC, no change; n.d., not determined; P, progressive disease; PR, partial response.

aspirates could be obtained from most of the patients, so that we might have some false-negative results. On the other hand, we cannot exclude that tumor load is below our sensitivity of tumor cell detection, especially in group 1 patients. Furthermore, although enrichment of tumor cells was performed in most cases, the results obtained in our group in cell culture models differ from those in clinical samples. This might be explained by the construction of the beads carrying only one antibody against one epitope on tumor cells, so that because of the antigenic heterogeneity of tumor cells, especially in breast cancer, tumor cells expressing other epitopes will not be caught by our technique. In the future, "cocktails" of beads, directed against a variety of antigens, may overcome this problem. Nevertheless, the presence of CK+ cells after therapy in group 2 patients indicates that tumor cells can survive HD chemotherapy.

In patients with metastatic disease, high numbers of CK+ cells were also shown in studies by Cooper *et al.* (34), who found 60–80% of microscopic tumor in histologically normal BM harvests. In this study, as well as in our setting, the majority of patients developed progressive disease in prior metastatic sites, which suggests that inadequate cytoreduction rather than seeding of new tumor sites was the reason for treatment failure. Thus, CK positivity of the BM after therapy may be suitable to predict response to systemic therapy.

Tumor cells still present after chemotherapy may as well be a result of reinfused PBSCs contaminated with tumor cells. To address this question, we also analyzed the PBSC samples for circulating CK+ cells. The overall detection rate in both patient groups was 27% (9 of 33 patients), with the highest probability being documented in patients with metastatic breast cancer. Similar detection rates for tumor cell contamination in PBSCs were found by three other groups studying CK+ cells in PBSCs (25, 27, 35). In contrast to studies with BM, the relationship between tumor cell contamination of PBSCs and its clinical significance has not been extensively investigated. Whereas Sharp *et al.* (26) showed that occult tumor cell contamination of PBSCs conferred a worse prognosis, Stadtmauer *et al.* (27) demonstrated that contamination of PBSCs was not correlated with decreased survival. The latter findings are in accordance with our data for group 1 patients with contaminated PBSC collections but no evidence of disease after a follow-up time of 39 and 32 months, respectively. In addition, the same tendency could be shown in two of seven group 2 patients who are still alive after a follow-up time of 33 and 38 months, respectively.

Although progenitor cell collections were not analyzed for clonogenic tumor cells, it appears likely that not all of the tumor cells detected by IC are able to grow *in vitro* (36). Furthermore, it is not known whether tumor cells in PBSCs can survive after cryopreservation in DMSO (10%). In addition, host immune factors may be important in eliminating small numbers of residual tumor cells.

In some other studies, the role of contaminated PBSC collections in stage IV breast cancer patients is discussed controversially. One study (37) supports the hypothesis that reinfused cells that were not exposed to HD chemotherapy and did not develop multidrug resistance may contribute to relapse. In contrast to these studies, Cooper *et al.* (34) postulated that the degree of tumor cell contamination may be rather a biological marker of residual tumor burden than contribute directly to relapse. Alternatively, this group postulated that there could be a threshold above which reinfused tumor cells confer a worse prognosis.

Data by Ross *et al.* (36) support the current opinion that PBSC collections may be preferred to BM as source of hematopoietic stem cells for autologous transplantation because of a lower degree of tumor cell contamination. This also holds true for our trials that identified 2 of 3 CK+ BM patients in group 1 and 9 of 15 CK+ BM patients in group 2 with no CK+ cells in their stem cell grafts. Only two patients without immunocytochemically detectable BM involvement (patient 12 in group 1 and patient 7 in group 2) had PBSC micrometastases. Thus, only in some instances, BM may provide a less contaminated source of hematopoietic stem cells.

The heterogeneity of solid tumors (different cell-cycle phase; mechanisms of resistance) poses a problem for all kinds of therapy and limits the chance of complete elimination of all of the residual tumor cells. The success of chemotherapy, aimed at proliferating cell populations, may be limited by the fact that many of the residual systemic tumor cells may be nonproliferative or dormant (16). In this context, Braun *et al.* (17) showed the heterogeneity of antigen expression in breast cancer, resulting in CK+/17-1A+ and CK+/Her-2/neu+ cells present in the BM before therapy. Furthermore, the persistence of isolated tumor cells in the BM after chemotherapy with taxanes and anthracyclines in high-risk breast cancer patients was shown to be an independent predictor for reduced overall survival in a multivariate analysis (14). Our study provides a first insight into the changes in the pool of micrometastatic cells during HD or intensified chemotherapy. We clearly demonstrate that micrometastatic cells survive even high doses of chemotherapy as demonstrated by the analysis of PBSCs and BM. Furthermore, most of the residual cells express the immunotherapeutic antigen 17-1A on their surface. Her-2/neu, as one further candidate for antibody-based immunotherapy, was not analyzed on these residual cells but has been demonstrated to be present on breast cancer cells before chemotherapy (16, 17). In addition, overexpression of Her-2/neu before chemotherapy was associated with partial response or progressive disease, and it has been suggested that blood-borne Her-2/neu-CK clustered cells are the possible precursors of distant metastases (38). In our study, the role of Her-2/neu could be demonstrated by serum analysis of Her-2/neu. Although enhanced levels could be demonstrated in patients with metastatic disease before chemotherapy, the measurement of Her-2/neu in serum samples is not able to detect residual disease after chemotherapy.

Thus far, however, the first encouraging results of Phase II trials with HD chemotherapy have not been confirmed in randomized studies. Whereas Stadtmauer *et al.* (3) and Rodenhuis *et al.* (4) could not show any benefit of HD chemotherapy as compared with conventional treatment in patients with metastatic disease, this issue seems to be not yet definitely resolved in patients with locally advanced disease, and the final results of ongoing trials must be awaited. Although the use of HD chemotherapy with PBSC support has been questioned recently, follow-up and prospective clinical trials will have to prove the clinical relevance of micrometastatic cells in PBSCs and BM even after HD chemotherapy. Nevertheless, in upcoming clinical trials, the characterization of residual cells might help to identify those patients who could benefit from additional cell cycle-independent treatment protocols. Among several proposals, antibody-based immunotherapy targeting the 17-1A antigen or Her-2/neu has been proposed recently as an effective treatment in breast (21, 22) and colorectal cancer (23). Furthermore, purging of stem cell grafts from breast cancer cells resulted in effective elimination of tumor cells (24, 25).

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